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CIBA FOUNDATION SYMPOSIUM

Jointly with

THE INTERNATIONAL UNION OF BIOLOGICAL SCIENCES

ON

BIOCHEMISTRY OF HUMAN GENETICS

Editors for the Ciba Foundation

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and

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THE Ciba Foundation, a unique international institution, owes its inception to the generosity of CIBA Limited, Basle. However, being established under British trust law, it enjoys complete independence in practice and policy.

Under the guidance of its distinguished Trustees, the Foundation offers accommodation to scientists from all over the world at its home in Portland Place. Foremost in its activities is the organization of small conferences, the proceedings of which are published in book form in the manner of the present volume. The Foundation convenes many other informal discussions between research workers of different disciplines and different nationalities and each year invites an outstanding authority to deliver a special lecture. An exchange programme between French and British postgraduates is conducted and a library service is available. Furthermore, the Ciba Foundation attempts in every other way possible to aid scientists, whether they be Nobel Laureates or young graduates making their first original contribution to research.

The purpose of the Ciba Foundation, which is to promote international co-operation in medical and chemical research, is symbolized in the armorial bearings by five interlaced rings representing the continents, a black sacrificial cock (emblem of Aesculapius) holding a medical caduceus, and three regular hexagons for chemistry. Its domicile in London is indicated by the red sword of St. Paul and the British lion; the wyvern and the crozier, symbols associated with Basle, refer to the sponsoring firm located in this ancient Swiss town.

PREFACE

As part of a special programme in the year of the early but significant milestone of its 10th Anniversary, the Ciba Foundation has organized for the first time conferences in places other than its own agreeable and well-equipped premises in London.

The first of three such conferences, on the small scale and in the informal style characteristic of the Foundation, was arranged in Naples, Italy. This volume contains the papers and discussions contributed by the group happily gathered in a place where so many generations of mankind have left their traces and where their living descendants are so humanly helpful and hospitable.

This symposium on human biochemical genetics was initiated by Professor Montalenti on behalf of his own Department at Naples and also of the International Union of Biological Sciences. The Rockefeller Foundation also generously supported the project. Professor Montalenti and Professor Siniscalco made many of the preliminary arrangements in Naples and played vital parts in the successful realization of the meeting.

The young Ciba Foundation was also most fortunate in holding its first overseas conference in the world-famous, almost centenarian Zoological Station, and received every conceivable assistance from Professor R. Dohrn and Dr. Peter Dohrn.

A preface is quite inadequate for acknowledgements where so many are so richly due and warmly felt, but the Editors must mention also their thankfulness to Professor Siniscalco, Dr. Harris and Miss Joan Etherington for detecting the threads of discussion among the simultaneously recorded street sounds of Naples.

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S. Brenner	Medical Research Council Unit, Cavendish Laboratory, Cambridge
Sir Macfarlane Burnet	Walter & Eliza Hall Inst. of Medical Research, Royal Melbourne Hospital, Melbourne
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L. Califano	Inst. of General Pathology, Naples
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H. Harris	Dept. of Biochemistry, The London Hospital Medical College, London
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H. Kalmus	The Galton Laboratory, University College London
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J. Lederberg	Dept. of Genetics, Stanford University Medical School, Stanford, Calif.
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A. Monroy	Dept. of Comparative Anatomy, University of Palermo
G. Montalenti	Inst. of Genetics, University of Naples
W. T. J. Morgan	The Lister Inst. of Preventive Medicine, London
J. V. Neel	Dept. of Human Genetics, University of Michigan Medical School, Ann Arbor

L. S. PENROSE	The Galton Laboratory, University College London
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WINIFRED M. WATKINS	The Lister Inst. of Preventive Medicine, London
S. W. WRIGHT	Dept. of Pediatrics, University of California Hospital, Los Angeles

ADDRESS OF WELCOME

L. CALIFANO

Il Consiglio delle Ricerche vede con la più viva simpatia questo Symposio della Fondazione Ciba, che si svolge a breve distanza dall'altro affine tenuto presso l'Accademia del Lincei, sotto gli auspici dello stesso Consiglio. Il fatto che il Simposio di Genetica Biochimica si svolga presso la Stazione Zoologica è assai significativo. Questo Istituto fu fondato da Anton Dohrn per la ricerca zoologica sistematica ed ecologica, per la citologia, istologia ed embriologia e fisiologia delle forme viventi del mare, ma il fine reale supremo che animò l'impeto creativo del suo fondatore è quello della ricerca della evoluzione e della azione di dette forme. La lapide apposta sulla facciata dell'Istituto esprime nelle armoniose frasi scritte dal Croce tale destino: "quanta luce di verità promettesse l'indagine della vita nei mari". La Stazione Zoologica nella sua lunga vita ha potentemente contribuito a tale indagine ed ha così ben meritato nella sua vita. Il grande spirito di Anton Dohrn aleggia qui in questa sala dove è anche ritratta l'effigie della persona e si coglie volentieri anche questa occasione per rendere omaggio e rispetto alla sua memoria con grato animo, come gratitudine e rispetto si esprime al paziente ed umano continuatore dell'opera paterna, il caro Rinaldo, che ha tenacemente retto le sorti dell'Istituto per oltre trenta anni, ed augurio ed incoraggiamento si rivolge a Pietro, erede di sì nobili tradizioni, che imprime all'Istituto con sovrumana fatica un nuovo corso, quale è richiesto dal progresso e dalla specializzazione della biologia. Per molti anni è sembrato che la Stazione Zoologica poco potesse contribuire allo sviluppo della genetica, e solo potesse in tal campo portare contributi collaterali. Quando invece dalla genetica formale si è sviluppata la genetica biochimica, l'ufficio della Stazione Zoologica è apparso di nuovo importante. Dallo studio delle forme e delle strutture e dei meccanismi che li determinano, ha avuto qui

una sua culla la indagine biochimica della vita dei mari che riserba grandi possibilità di scoperte, e poichè ogni fatto biochimico, cioè ogni funzione metabolica, energetica e plastica è di dipendenza genica, lo studio comparativo e biochimico sarà di grande significato per la genetica chimica e per quella che si dice l'evoluzione biochimica, e certamente per la evoluzione delle forme, che è strettamente connessa. Perciò l'odierno Simposio di Genetica Biochimica per la Stazione Zoologica ha lieto auspicio per il fervore dell'opera futura. Il Consiglio delle Ricerche e la Stazione Zoologica sono così molto grati al caro illustre amico Prof. Montalenti, che ha con saggezza lungimirante promosso questo Simposio, egli che alla sapienza ed al fervore di ricercatore unisce passione sincera per il progresso degli studi, e perciò anche grande amore per la Stazione Zoologica. E ciò lo testimonia anche la attuale premura a che il Simposio della Fondazione Ciba di Genetica Biochimica si svolgesse in questo Istituto.

CHAIRMAN'S OPENING REMARKS

G. Montalenti

Before the work of the symposium begins, I should like to explain its meaning and how it originated. This I shall do especially for the benefit of the invited audience present at this opening session, and I hope that the members of the symposium will forgive me if I go into some details which may seem quite trivial and obvious to them.

In 1949 a symposium was held in Milan at the Istituto Sieroterapico Milanese on "The Recent Contribution of Human Genetics to Medicine". In my opening address I stated something which was quite obvious at that time, i.e. that Man, who had formerly been considered as the least suitable animal for genetical research, had gradually proved to be one of the best subjects for at least some kinds of genetical investigation.

It was long thought also that human genetics was merely an applied branch of biology. Fundamental problems, it was said, have to be studied in animals, in plants, in micro-organisms, if the general principles are to be worked out; as soon as these are discovered, one can try to see whether they hold true in Man, and how they can be applied to explain hereditary or evolutionary patterns in this particular organism. As investigation on human genetics proceeded, however, it was realized that this was not true, or at least it was only a part of the picture. Human genetics is no longer a mere applied science; on the contrary, human beings are a very good subject for the investigation of some primary basic genetical or evolutionary problems. For instance, as Haldane pointed out at the 1949 symposium in Milan, human populations are perhaps more suitable than any others for the investigation of problems of variation of gene frequencies, whether due to selection or to other causes. The selective value of some genes—such as those responsible for sickle-cell

anaemia and thalassaemia—and some blood groups have been demonstrated or are under investigation and appear to offer a promising field of study.

Only a decade has elapsed since that meeting, and the situation has since changed quite considerably. Another kind of fundamental problem has been focused by human genetics and has led already to fine results, i.e. the problem of how genes act, which is clearly an essentially biochemical one.

It is often said that biologists are now busily rewriting all their knowledge in terms of biochemistry. It must be added, however, that just as there are two ways of dealing with biological phenomena, namely pure description and interpretation of the causal relationships, the same is true of the chemical presentation of biological events: it may be merely descriptive, or it may try to penetrate more thoroughly and intimately into the processes, i.e. to discover the elementary molecular reactions and the basic principles whereby a chemical fact is transformed or amplified giving rise to a biological event perceptible at the phenotypic level.

Genetics has greatly contributed to the chemical interpretation of basic biological phenomena. Indeed it is mainly thanks to the genetical outlook that we have a deeper insight into, and are perhaps approaching the solution of some of the most fundamental biological problems, such as specificity, reproduction—which implies transmission of information—and *quod est in votis* the origin of living matter.

Taking into consideration only biochemical genetics as such, it is well known that the first work which had a considerable impact by arousing a lively interest among geneticists was that of Ephrussi and Beadle, in the nineteen-thirties, on substances controlling eye colour in *Drosophila*. Then came Beadle and Tatum's work in *Neurospora*, which established the fact that a gene may control a single step in a given biochemical reaction, thus providing a clue for the investigation of the mode of action of genes. However, it is now generally recognized that this very fact had been discovered as early as 1902 in Man. At that time, in the infancy of genetics, Garrod

found in Man a beautiful instance of what he very properly called "inborn errors of metabolism": namely, alcaptonuria. Garrod demonstrated that this anomaly, characterized by excretion of alcapton in the urine, is the result of a block in the chain of reactions by which the amino acids, phenylalanine and tyrosine, are metabolized. Many other instances were described and elaborated in subsequent years in a masterly way by the same author with the help of Bateson's advice on genetical aspects. Each instance seemed to be due to a single Mendelian factor—a gene. Garrod saw clearly that the normal gene controls the very step of the reaction which in the presence of the mutated allele is stopped or altered. Garrod is undoubtedly to be considered as the founder of the one gene–one enzyme theory, and he also had a clear idea of the biochemical polymorphism in human populations. Garrod's discovery and theoretical outlook have been fully understood and appreciated only with a delay of some decades.

It may be said, therefore, that the first important step in the understanding of gene action was made in human genetics. Later came the beautiful work on *Neurospora* and other micro-organisms which led, as is well known, to most brilliant results, and provided further evidence in support of the one gene–one enzyme hypothesis which has played an important rôle in genetics.

The next achievement along this line was the discovery by Pauling, Itano, Singer and Wells in 1949, that in sickle cell anaemia the red cells have an aberrant haemoglobin called haemoglobin S, which has an electrophoretically different behaviour from normal haemoglobin A. According to the finding of Neel, sickle-cell anaemia depends on a gene pair. Pauling and co-workers were able to show that homozygous individuals have homogeneous haemoglobin of an aberrant nature; heterozygotes, showing the so-called sickle-cell trait, have a mixture of normal and aberrant haemoglobin: about 60 per cent of the former and 40 per cent of the latter. Normals have only haemoglobin A. In this particular case it appears that the action of the gene is not to build an enzyme. It gives

specificity to a non-enzymic protein, which may probably be considered as the primary product of the gene action. If this is so, we are coming close to an understanding of the function of the gene at its very beginning. Correspondingly, the one gene-one enzyme hypothesis had been modified to that of one gene-one primary function.

Recently Ingram has been able to go a step further in this analysis, showing that the primary effect of the mutation in the case of sickle-cell anaemia consists in the substitution of a single amino acid by another in a defined position along one of the polypeptide chains of the molecule of haemoglobin. The primary action of the gene here is to bring about a comparatively simple chemical change.

Now if we look at the main trends in the analysis of genetic material—the idioplasm as our grandfathers used to call it—trends which have been recently summarized in a masterly way by Pontecorvo, we find that they are, *grosso modo*, two: (1) structural analysis carried out by genetical means, and in two directions, i.e. down towards the molecular level, whereby the old concept of a gene as a recombination and functional unit vanishes and is replaced by what has been called a cistron, built up of a definite sequence of subunits or sites; and upwards, trying to explore upper levels of integration in the chromosomes, of which so little is known; (2) chemical analysis, trying to find out how the molecular specificity is expressed, how it is coded, and how the code works in letting its cryptography become clear, by building exactly those molecules for which it carries the information.

From the facts which I have briefly summarized it appears that some findings of human genetics have a bearing on this last part of the problem. They may help to tackle the problem of the mode of action and hence of the structure of the gene. In some respects it may be said that Man is not less favourable than micro-organisms or *Drosophila*. This may be surprising if we think of the complexity of Man as compared to bacteria, and of the difficulties of working out his genetics, as compared to *Drosophila*, but it is mainly due to the fact that our

knowledge of physiology, pathology and individual differences is far more advanced in Man than in any other organism. For this reason we may hope that other problems, such as allelism and gene structure at the genetical level, may also be studied in Man.

It seemed advisable, at this point, to gather together people interested in these problems so that they might summarize and discuss the findings and consider future lines of research. The Ciba Foundation was approached, and made an enthusiastic response to our proposals. The symposium was financed in part also by the International Union of Biological Sciences, and by a grant from the Rockefeller Foundation.

The programme has been arranged as follows: after an introduction on genetical analysis in Man, a first section is devoted to some problems of genetical control of metabolism. This is always one of the main sources of information, and new facts collected, even if merely descriptive at the beginning, are always welcome, as is any refinement in the elaboration of already known phenomena. The second main section is devoted to the field I have stressed as one which has already given a good yield and is still very promising, namely the synthesis of proteins and other macromolecules, e g. the blood group mucopolysaccharides. In the third and final section, basic facts relevant to the mechanism of gene action will be discussed.

Since most of the work on human biochemical genetics has been carried out on substances which are present in the blood or in urine, our knowledge is very wide and deep in these fields, whereas we know almost nothing of what happens in other tissues or organic fluids. This is due, no doubt, mainly to the ease with which blood and urine may be collected and analysed. It is more than likely that the careful examination of other tissues would lead to important findings. One of the methods of approach to genetical problems at this level is tissue culture, and it is hoped that this method may enable us to study some fundamental aspects of genetics, even that tissue culture in the hands of geneticists will eventually

provide a clue for the investigation of that intermediate field, between embryrology and genetics, which includes the baffling problem of embryological differentiation. Therefore, it was thought advisable to include in this symposium a panel discussion on the tissue culture approach to genetical problems.

Why were Naples and its Zoological Station chosen as the site of this meeting? Naples itself was chosen because some of my co-workers at the Istituto di Genetica have become interested in problems of human biochemical genetics, i.e. thalassaemia, the genetics of plasma proteins, and primaquine sensitivity or favism, for the study of which the Italian population offers particularly good opportunities. Our research workers are very happy to enjoy the possibility of having a discussion here on the problems which I have mentioned, and of personally becoming acquainted with many of you. We have asked the Director of the Zoological Station for hospitality because the rooms available at the Istituto di Genetica were not convenient for holding the meeting. This, as you know, is an international laboratory in which a great deal of outstanding work in biochemistry has been performed in the past and is being carried on at present. Moreover, the Zoological Station was established by Anton Dohrn almost a century ago, under the stimulus of the new evolutionary doctrines and with the principal declared aim of promoting research in any field of biology, in the light of the theory of evolution. Thus, students of evolution as we ultimately are, it is very appropriate that we should gather in these rooms.

GENETICAL ANALYSIS IN MAN

L. S. PENROSE

The Galton Laboratory, University College London

Intrafamilial likeness, measured by correlations between relatives

Human genetics is an applied science. It makes use of techniques of all kinds as they become available for the study of the hereditary processes in Man. Chance enters into these processes as an integral component so they must, to some extent, be analysed statistically.

The most obvious value of a statistical technique such as correlation, which was introduced by Galton (1889) and improved by Pearson (1909), is to deal quantitatively and precisely with traits which do not segregate. Of course it can be used with segregating traits if desired but its use is less necessary if the effects of genes are distinctly visible. In the first enthusiasm for using the newly invented technique, large amounts of data were collected on physical measurements for parent and child pairs and for sibs but it was soon realized that the results were not very informative. In spite of the intriguing algebraic account of the effects of dominance and assortative mating on correlation values by Fisher (1918), and the extension of the theory by Hogben (1932) to include sex-linked genes, analysis of quantitative genetical data in Man has become less and less popular. Indeed, it is remarkable how little work has been done on the genetics of graded traits for the last thirty years in spite of the obvious practical value of knowing the ranges and distributions of measurements of the offspring of different kinds of parents. Actually quantitative analyses can give useful clues to underlying effects of single gene pairs besides supplying hints about the relative significance of nature and nurture. Typical recent examples are

Holt's (1955) analyses of dermal ridge counts, traits strongly genetically determined, and birth weight, a measurement influenced by a great variety of environmental and hereditary factors (Robson, 1954) (see Table I).

Table I

APPROXIMATE PARTITION OF CAUSES OF VARIATION OF BIRTH WEIGHT BETWEEN HEREDITARY AND ENVIRONMENTAL CAUSES

Maternal genotype	0·20
Maternal environment (general)	0·18
Maternal environment (immediate)	0·06
Maternal environment (age)	0·01
Maternal environment (parity)	0·07
Foetal genotype (without dominance)	0·15
Foetal genotype (dominance)	0·01
Foetal genotype (sex)	0·02
Unknown uterine environment	0·30
Total	1·00

In certain restricted circumstances, the correlation coefficient between relatives can be most informative. For example, we may want to know whether variation of an abnormal dominant trait in a pedigree is caused by environment, by independent genes, autosomal or sex-linked, or by interaction with the neutral allele. This general idea, which was introduced crudely by Haldane (1941), can be used to establish a given hypothesis with remarkable clearness in certain cases; in particular, modification, caused by members of an allelic series, gives rise to a strong correlation for affected sibs and zero correlation for parent and child. The effect was first observed by Goldschmidt (1938) in pedigrees of dystrophia myotonica and has since appeared in Renwick's (1956) analysis of nail patella syndrome and in Harris and Robson's (1959) pedigrees of hypophosphatasia. It comes about because the same allelic partner gene cannot be transmitted from affected parent to affected child. The method can easily be applied to abnormal haemoglobins and other biochemical traits.

In general, however, the use of correlation is only a preliminary method of investigation. The modern aim is to break down continuous variation into its single factor components.

Technical methods of family study

(a) **Segregation:** When traits segregate well, as was found long ago in the blood antigens, we divide the population into affected and unaffected classes and new vistas for genetical investigation are opened. Mistakes in classification are often made even when there is good separation of phenotypes and it is at last becoming an accepted practice to check every qualitative judgment by actual measurement. When a trait can be expressed as a quantitative chemical distinction, the result is usually far more satisfactory than if it is merely classified as present or absent. It is, thus, a general methodology in human genetics, first to replace qualitative judgment by measurement and, secondly, to measure a trait which is as close as possible to the gene. For example, it is better to use objective reflectance measurements of hair and skin than to be content with classifying people subjectively as albino, fair, dark, and so on. It is better, again, to measure the amount of melanin in tissues and still better to measure the quantities of enzymes necessary to the formation of melanin.

The first approach, changing a qualitative judgment into measurement of the concentration of abnormal metabolites, proved particularly useful, e.g. in understanding the genetics of cystinuria by enabling homozygous and heterozygous types to be sorted out. Even more striking is the direct identification of enzyme deficiency in the living subject, in heterozygotes as well as homozygotes, with inborn chemical errors, such as galactosaemia. From the genetical point of view, the ascertainment of the precise degree of overlapping (if any) of the normal and abnormal groups is a most useful datum.

(b) **Consanguinity of parents:** When genotypes have been shown to segregate, the next step is to discover their familial distribution. One of the earliest tests was the

occurrence of parental consanguinity in affected sibships, recognized by Bateson and Saunders (1902) to be the hallmark of a rare recessive trait. This is an objective fact, clear cut and irreversible.

Cousin marriages can also be used, in reverse, as potential concentrated sources of homozygous offspring. The method has been successfully exploited recently by Neel and his colleagues on the investigation of foetal abnormalities in Japan (Neel, 1958). The argument can be used, as for example by Slatis and Reis (1957), in the attempt to find out how many heterozygous loci for deleterious recessives are carried by the average person.

Formal arguments from statistics on offspring of cousin marriages are unfortunately rather unreliable for a variety of reasons. We do not know how many recessive traits are lost at a stage too early to record. Moreover, cousin marriages are distributed very unevenly in the population and rates of inbreeding are altering rapidly everywhere. In actual work, moreover, traits not recessively caused are liable to be wrongly included. Furthermore, although, in any disease, a high parental consanguinity rate marks it as a rare recessive condition, it is probably inaccurate to estimate the general population frequency of heterozygotes from this observation because genes are unevenly distributed, as in pentosuria, Wilson's disease and amaurotic idiocy of infantile type.

A largely unexplored field concerns consanguinity in father's or mother's parents. Homozygous states in the mother, so indicated, are especially likely to be interesting.

(c) **Search for incomplete recessivity:** A most characteristic feature of modern human genetical results is the finding of traits in heterozygotes hitherto unsuspected. Some twenty-five years ago, almost the only case where heterozygotes could be distinguished from both homozygotes (apart from AB and MN blood groups) was the famous Mohr and Wriedt (1919) pedigree of brachyphalangy. Now the position is reversed and it is not respectable to discuss a homozygous trait without mentioning the findings in carriers.

One of the earliest important advances here was the statistical proof, by Hsia and co-workers (1956), that carriers of phenylketonuria had, on the average, elevated levels of blood phenylalanine and that the difference was especially noticeable in a stress situation produced by phenylalanine feeding. There was, nevertheless, plenty of overlap between carriers and normal homozygotes. As in cystinuria, we may suspect that the variability in heterozygotes points to a variety of alleles or even to independently located genes having similar phenotypical effects. We shall hear much more about this kind of thing in the present symposium, especially in relation to galactosaemia and other states, where the trait studied is closer to the gene than the quantity of a given metabolite.

It is satisfactory to note that the nearer we approach to the enzyme, our genetical entities become more accurately definable. As already mentioned, quantitative traits, formerly much studied, are far from the genes in the sense that many steps, environmental, physiological or genetical interactions, intervene. It is pleasant to be able to salvage out of the sea of continuous variation significant contributory causes due to single gene action, like hair colour dilution in phenylketonuria (Cowie and Penrose, 1951) and, as shown by Siniscalco and co-workers (1959), alterations in physical type caused by microcythaemia.

(d) **Population genetics**: In order to explain the prevalence of genes in any given human population, we must understand their relation to biological fitness. Human generations are long and gene frequencies move slowly but, if there is strong selection against any genotype, its frequency must inevitably tend to diminish. Some anthropologists like to distinguish between normal and abnormal characters but this is not an easy distinction to justify. Any gene may be abnormal in a particular environment. In this connexion, it is noteworthy that entirely unsuspected genetical sensitivities to artificial new environments produced by synthetic drugs have been brought to light. Generally speaking, however, it is reasonable to assume that any gene found persistently in

human populations is likely to be in a fairly stable state with respect to selective forces; that is to say, in the past it has neither rapidly been eliminated nor rapidly become universal else we should not have been able to study it. This is one of the principles which was established in human population genetics in consequence of the theoretical investigations of Fisher (1930), Haldane (1932) and Wright (1931) some thirty years ago.

After a long latent period, the search for a balance between selection and mutation has become an integral part of the description of every hereditary trait, normal or abnormal. With severe dominant diseases, which appear without any precursor in a significant proportion of pedigrees, there is no obvious contradiction in assuming that they are caused by fresh mutations. The uncertainties in this assumption lie with the clinical classifications of cases rather than with theory. For sex-linked traits, arguments of the same type are also cogent. But, concerning recessives, the matter was altogether different. On the same theoretical basis we would have to assume mutation rates per chromosome per generation approaching 1 per cent in extreme examples, like sickle-cell trait and cystic fibrosis of the pancreas. It was not unnatural to look elsewhere for sources of equilibrium, and heterozygous advantage over both homozygotes seemed the obvious explanation. After much argument, the sickle-cell haemoglobin seems to be assuming its place as an antimalarial substance. The position of glucose-6-phosphate dehydrogenase deficiency, as ascertained by Motulsky and his co-workers (1959), may be stronger. This situation is particularly intriguing because the female heterozygote must be best fitted to survive the double attack by natural selection expressed, on the one hand, in malarial infection and in haemolytic disease, caused by faulty diet, on the other.

The methodology of measuring fitness in human phenotypes is, so far, in a chaotic state. A direct count of offspring of selected phenotypes is seldom likely to be rewarding. The sources of error in collecting data and the ranges of family

size are enormously large compared with the magnitudes of the effects to be demonstrated. This difficulty applies especially to the search for, say, an increase of something like 2 per cent in the fitness of heterozygotes, as compared with the general population. A small increase of this kind is necessary and sufficient to keep an ordinary, not too rare, lethal in circulation without bothering about mutation. In practice, the method of comparing the incidence of a trait in different age groups of a population gives the most convincing data about the biological fitness with which it is associated.

Much more could be said about that subject and upon the unreliability of mutation statistics. Let it suffice to emphasize that, at the present time, there is great ignorance concerning the frequencies of hereditary diseases in populations. The first steps to obtain accurate frequency figures can be expected to be made *whenever a biochemical or equivalent test becomes* standardized as happened, for instance, in the work of Hirszfeld and Hirszfeld (1919) in the case of the blood antigens and is now a practical proposition with many serum proteins.

The human chromosome map

(a) Use of tissue culture methods: The study of linkage in Man has for long been regarded as a useless and erudite activity only pursued by cranks. True, there are now three well-established pairs of autosomal loci in proximity on three different chromosomes. On the X-chromosome the task is easier. Provided that we know they are sex-linked, we have only to find out how close is the linkage between loci concerned. The map distances for haemophilia, colour blindness, muscular dystrophy, agammaglobulinaemia and the sex-linked enzyme deficiency should all be known fairly soon. However, there are new actors coming on to the stage in this drama, and much will soon be learned about autosomal gene location from studying cultures of human cells and examining the chromosomes of abnormal individuals. The ideal state would occur if *there should be an observed deletion* and a corresponding

character in the person who carried it, comparable with what would be expected in an individual hemizygous for a gene at that locus. Absence of one whole chromosome can give similar information about all the genes upon it. The absence of the Y-chromosome has been found to cause absence of maleness (Ford *et al.*, 1959*a*), for example. These favourable situations will, of course, only occur in rare circumstances but the constancy of chromosome picture (karyotype) in conditions of culture over fairly long periods, first demonstrated by Tjio and Puck (1958), is most encouraging. Cultures from tumours, especially of leukaemias, are likely to provide excellent data. The natural history of changes in the blood cells must be intensively studied in order to control the disease and, at the same time, genetical facts will come to light, sometimes inadvertently, placing an antigen locus on a particular autosome. This may happen when an antigenic peculiarity in a patient is first correlated with an altered karyotype.

(b) "**Mongolism**": The example of mongolism is worth special mention because, although trisomy (Ford, *et al.*, 1959*b*) is not so genetically informative as haploidy, we may still learn something about gene action here, perhaps just by re-examining already ascertained facts.

Among outstanding morphological peculiarities are the dwarfed stature, shortened extremities, simply convoluted brain, fissured tongue, round head and unusual palmar markings. These might be caused by some generalized alteration in cell size or water content. There might be generalized changes in cell metabolism caused by the presence of too much of some proteins. The puzzle as to what three doses of a programme may do to an individual is, to some extent, elucidated by Stern's (1943) work on triplo IV in *Drosophila melanogaster*. A gene locus on this very small chromosome controls the formation of a wing vein. Stern found that the trisomic condition had a slightly distorting effect but that, in spite of this, the series of characters due to different alleles was preserved. He was dealing, of course, with secondary non-disjunction here but, in the mongol, the origin must be primary non-

disjunction in a maternal ovum in the majority of cases. If this took place in the first meiotic division the result would be to make the child twice as like its mother as its father for any factor determined by genes on the triploid chromosome. This gives us an immediate test for genes located upon it.

Applied to the position of the "t" triradius on the palm it gives an interesting result. This character is an inherited quantitative trait transmitted, in the normal population, equally by father and mother. The mongol, however, is significantly more like its mother than its father in respect of this character (Penrose, 1954*a*). The difference is detectable although intrafamilial correlations between mongols and relatives are all lower than those between normal relatives, probably because of the distortion caused by trisomic dwarfing. It is not certain, however, that this result should be taken at its face value. Examination of blood groups in families where mongols have occurred shows that, for the ABO antigen system, and possibly others, the mongol child resembles its mother rather than its father. Increased likeness of child to mother would also occur if the mother was more often homozygous than is usual in the general population. Homozygosity in a parent is known to be a cause of chromosome aberration (Rees, 1955), so that there is another explanation of increased likeness between mother and child to be carefully considered. The lack of conclusiveness in such results is, however, due to paucity of information and this can be remedied. It is, I think, likely that dosage effects of gene action can be accurately studied once a locus has been established on trisomic autosomes. Moreover, the effects arising from primary or secondary non-disjunction should be distinguishable genetically.

The fascinating associated problem of how maternal age (Penrose, 1954*b*) fits into the picture is worthy of consideration here. Birth order and paternal age have been proved insignificant by statistical analysis. The distribution of maternal age is such that it can be supposed that, for a subgroup containing more than three-quarters of mongols, i.e. about 78 per cent,

there is a risk which rises exponentially with maternal age (see Fig. 1). The primary non-disjunction in this subgroup might thus be attributed to an extracellular or intracellular chemical agent. Experiments on the genesis of triplo IV in *Drosophila* may perhaps provide the best approach to the problem. In the remaining subgroup, about 22 per cent, the maternal age distribution does not differ from that in the general population but there is a slight risk of recurrence in the sibship. Exten-

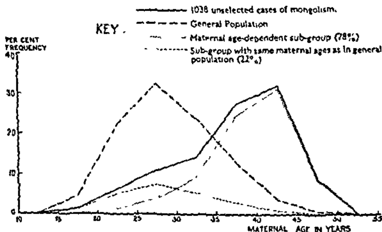


Fig. 1. Maternal age distributions. General population compared with cases of mongolism subdivided into two groups.

sive analysis of families in which more than one case has occurred has suggested that there is a special predisposition present in some mothers. This predisposition could be the effect of homozygosis or possibly even gonadal mosaicism leading to secondary non-disjunction which is independent of maternal age. Detailed discussion of these questions would take us too far from the problem of gene action. However, we are reminded of the new areas for investigation which are opening up at the present time. It is surprising how quickly the need for biochemical methods of thinking and experimentation becomes evident. The control of mitosis and meiosis must also have its origin in hereditary biochemical processes.

Summary

At the present time the application of mathematical methods is no longer a dominating factor. Biochemical methods are now in the ascendant.

The basic data of human heredity must still be family studies but emphasis is not laid so heavily as formerly on finding exact Mendelian ratios. If the immediate results of gene action can be identified, the statistical arguments become less cogent. Parental consanguinity retains its place as the most useful marker for recessive inheritance.

The advances in chemical genetics of Man, which have in many ways outstripped those made on experimental stocks of lower organisms, have been made by rapidly absorbing new techniques such as chromatographic and electrophoretic methods of analysing body fluids.

A new star on the technical horizon is tissue culture of human cells but it seems that advances in cytological methods are even more significant. Indeed, the identification of the direct line of causation from a genic structure, with known chromosomal location, to the corresponding visible character is now a practical aim.

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DISCUSSION

Smithies: Prof. Penrose, you mentioned the search for incomplete recessivity. I wonder whether you are advancing as a hypothesis that there is no such thing as a completely recessive gene and that one will always be able to find evidence of both genes in the heterozygote if one looks correctly.

Penrose: I feel it is a good principle to try to find these differences in the heterozygote, and it is extraordinary how successful that has been lately. Prof. Fisher asked me in 1935 to go through the literature to see whether there was any indication of this intermediate type of inheritance in Man; there was hardly any, but this was probably because investigations were not being made in the right place. If one looks closer to the gene, closer to the chromosomes, then one has a better chance of finding two substances instead of one in heterozygotes.

Smithies: Perhaps if we looked at the detailed biochemistry of heterozygotes we might find this, although if we were looking at some overall effect on the individual we might not see it.

Penrose: I agree, but Muller, more than twenty years ago, was very insistent that there was something specific about recessivity. He thought that it was a chemical thing and that one substance suppressed another. It was this idea that led people to think of a

kind of built-in mechanism where there were not two things but only one in the heterozygote.

Smithies: But if you have two substances, one of which is functional and sufficient and the second inactive, then in the absence of detailed biochemical examination the heterozygote will be indistinguishable from the normal homozygote.

Pontecorvo: I do not remember how many cases have been described in micro-organisms in which a mutant is absolutely negative, i.e. not producing the enzyme and not even producing a protein antigenically related to the enzyme.

Brenner: Of those mutants that produce no enzyme activity, one would estimate that those possessing no protein might be of the order of half of the mutants. I know of no published case, but I have heard of cases where there is no protein produced that could correspond to the normal enzyme.

Luria: It seems important to find out in every specific case whether what appears in the hemizygous condition to be a null allele affects the function of a normal allele in the heterozygous condition.

Buzzati-Traverso: In view of the parallel that Prof. Penrose has drawn between genetical problems in Man and in *Drosophila*, I should mention that in *Drosophila* one can show biochemically that heterozygotes for recessive alleles are always different from homozygotes for the corresponding dominant Also in a few plants which I tested it turned out to be the same. Perhaps in Man this could be looked into, using suitable tissues or body fluids.

Pontecorvo: With reference to the remarks of Prof Penrose, I think Muller was perfectly clear about this matter, when he suggested at least thirty years ago his terminology for alleles: hypomorph, hypermorph, amorph, neomorph, antimorph, etc. We could use the term "neomorph" for the abnormal haemoglobin alleles and "amorph" for the cases in which a mutant allele apparently produces nothing, and so on. In fact, on the matter of "dosage" there was the considerable work of Stern on "bobbed" in *Drosophila*. The detected phenotype was in this case very far from the actual primary, or presumed primary, action of the gene. Stern examined the effect of hypomorph alleles in individuals with one, two, etc., up to, I believe, five doses. In spite of the fact just mentioned, there was a corresponding effect on the phenotype which would approach normal with more doses of mutant recessive allele.

Penrose: The phenomenon was rather different in that dosage was measured by a morphological method; a larger dose did not necessarily mean more of an enzyme.

Kalow: As a pharmacologist, I am used to seeing variation in the response of individuals to drugs. Both environmental conditions and

inherited properties are responsible for this variation. I wonder whether a systematic attention to this variation might not become one of the best means of obtaining information on genes and their relationship to enzymes and other body constituents.

Penrose: It is obviously going to be a very favourable field.

Cavalli-Sforza: In the majority of cases there occurs probably the continuous type of variation, which is difficult to analyse beyond a certain point, but clearly there are certain cases where the variation seems to be discontinuous so that one can hope to pin them down to a single gene difference. There are examples of drugs, such as aspirin, isoniazide, or some types of penicillin and sulphonamides, for which data published in the literature seem to point to a discontinuity in the distribution of individual responses or, more exactly, of the individual speed of absorption, elimination or destruction. Such cases seem to be very promising from the point of view of genetical analysis, and are also of potential clinical significance.

BIOCHEMICAL GENETICS AS ILLUSTRATED BY HEREDITARY GALACTOSAEMIA

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THE topic dealt with in the present paper presents problems of a great variety. First of all, the mere existence and biological distribution of the sugar, galactose, is puzzling especially from the standpoint of an evolutionist. The difference

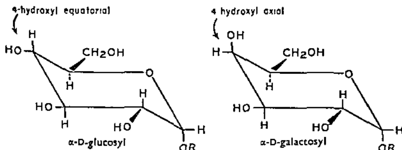


FIG. 1. Three-dimensional models of α-D-glucosyl and α-D-galactosyl compounds.

between glucose and galactose is confined to the carbon atom number 4 (see Fig. 1). The transition from glucoside to galactoside is essentially a kind of racemization between two configurations· the 4-hydroxyl in glucose occupies the equatorial position, whereas in galactose it occupies the axial position as related to the plane of the ring (Fig. 1).

It would be interesting to go deeper into the evolutionary

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aspects of galactose metabolism—the fact that the central nervous system and the milk gland have chosen to use galactose as one of their matrix elements. What could the positive vectors be? However, these positive developments will not be discussed here, but a different type of development —the existence of galactose-sensitive organisms. This phenomenon prompted us to enter the field of genetics.

First, because of the complexity of galactose metabolism in higher animals it is necessary to mention a particular chapter of carbohydrate metabolism, and introduce the various intermediary compounds which have been described in galactose metabolism. We are dealing here not only with free galactose or phosphorylated galactose but also with nucleotide hexoses, uridinediphosphoglucose (URPPG) and uridinediphosphogalactose (URPPGal). This important discovery we owe to Leloir (1951*a*, 1955).

There are three major steps in the Leloir pathway, catalysed by:

(1) Galactokinase: Gal + ATP → PGal + ADP
(2) Gal-1-P uridyl transferase: PGal + URPPG ⇌ PG + URPPGal
(3) UDPGal 4-epimerase: URPPGal ⇌ URPPG

The abbreviations used in this equation are meant to facilitate the understanding of mechanism. Thus, galactose-1-phosphate is abbreviated to PGal in order to illustrate that the transferase catalyses the "trading" of PG (glucose-1-phosphate) for PGal in the uridine nucleotide.

In animals, the enzymes catalysing these reactions are largely or exclusively constitutive but unequally distributed among various tissues. In micro-organisms, these enzymes are largely adaptive, i.e. they require galactose as inducer.

The system serves two functions: (i) to enroll galactose as an energy source in general carbohydrate metabolism, i.e. conversion to glucose-6-phosphate, triosephosphate, pyruvate, etc.; (ii) biosynthesis of more or less complex galactosides (and also polyglucosides).

It must be borne in mind that most organisms, and certainly the higher animals, do not need any external supply of galactose for their growth and development. Whatever galactosides their tissues may contain can readily be made from glucose or from glucose metabolites through reaction (3), i.e. the 4-epimerase catalysed reaction. A block in galactose metabolism is therefore apt to cause interference largely due to accumulation of intermediary products rather than due to a direct deficiency. As a general rule, jamming of intermediary products of a pathway is brought about by a block in any of the steps with the exception of the first one. This question will be dealt with more specifically below.

A particularly interesting type of galactose-sensitive organism is the human galactosaemic. The human disease, congenital galactosaemia, is characterized by specific tissue lesions (cataract, fatty degeneration in liver, retardation of brain function), by excretion of galactose in the urine and, as shown by Schwarz and co-workers (1956), by an accumulation of a galactose-1-phosphate (gal-1-P) in the red blood cells. These aberrations occur only if the affected individuals receive galactose. If a strictly galactose-free diet is imposed on the individual from birth, the organisms are practically insured against the development of specific galactosaemic lesions. It is important here to bear in mind that, in mammals, the deposition of galactolipids in the central nervous system takes place exclusively after birth (Folch-Pi, 1955); and yet, institution of a galactose-free diet provides practically complete protection against the development of cataract and feeblemindedness due to galactosaemia (Holzel, 1959). This point is stressed here because it has been customary to refer to galactosaemia as a block in 4-epimerase (Waldenase block). If this were so, a postnatal galactose-free regimen would interfere with the deposition of brain galactolipids and, therefore, quite likely also with the development of normal intelligence.

An accumulation of gal-1-P in the red cells of galactosaemics exposed to galactose indicates the existence of an involved disturbance in the regulation of galactose metabolism

as, for instance, in diabetes mellitus, or alternatively a direct block in one of the enzymes of the Leloir system succeeding galactokinase. We preferred to focus our attention first on gal-1-P uridyl transferase, an enzyme the existence of which had been predicted by Leloir (1951*b*) and which was observed by us a few years later in extracts of galactose-induced yeast (Kalckar, Braganca and Munch-Petersen, 1953; Munch-Petersen, Kalckar and Smith, 1955). We had developed specific enzymic methods (Maxwell, Kalckar and Burton,

Table I

QUANTITATIVE COMPARISON OF ACTIVITIES OF ENZYMES OF GALACTOSE METABOLISM IN THREE CLASSES OF SUBJECTS

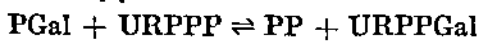
Classes of subjects	*Average activities, μM conversion/hr./g. cells (37°) (lysates of erythrocytes)*			
	Galactokinase	*Gal-1-P uridyl transferase*	*UDP-Gal 4-epimerase*	*G-1-P, PP-uridyl transferase*
Non-galactosaemics	0·10	4·8	0·32	>10·0
Galactosaemics	0·08	<0·02	0·35	>10·0
Galactosaemic carriers		2·0		

1955; Maxwell, 1957) for characterizing most of these steps and we felt, therefore, reasonably well equipped to try our luck with the solution of the riddle of galactosaemia. The enzymic techniques permitted us to demonstrate in an unambiguous way that the defective enzyme in galactosaemia is indeed gal-1-P uridyl transferase; this was first demonstrated in lysates of erythrocytes (see Table I). The other enzymes of the Leloir pathway were present (Kalckar, Anderson and Isselbacher, 1956*a* and *b*; Isselbacher *et al.*, 1956). Although this observation did clarify a number of problems, we were far from a solution since we had raised some new problems. The following are a number of problems which were to some extent clarified by this observation.

In view of the above discussion on the postnatal deposition of galactose in the brain, it is particularly noteworthy that 4-epimerase (Waldenase) is present in about the same amount in galactosaemics as in non-galactosaemics.

The transferase defect could also be demonstrated in blood from the umbilical cord of newborn infants (Anderson, Kalckar and Isselbacher, 1957). This concurs, of course, with the fact that the disease is an inborn error.

By the use of ^{14}C-labelled galactose, we were likewise able to demonstrate the same enzyme defect in liver biopsy samples from galactosaemic individuals (Anderson, Kalckar and Isselbacher, 1957).* In one case, we wondered whether we might have encountered an incomplete enzyme defect (a "leaky mutant", as the microbiologists call it). We found that this adult galactosaemic seemed to contain small amounts of transferase in his liver tissue but none in his haemolysates. I am inclined to think that this individual may rather have developed a related transferase which can incorporate gal-1-P in a somewhat different way into nucleotide. We had earlier described the existence of such a transferase in galactose-adapted yeast (Kalckar, Braganca and Munch-Petersen, 1953; Munch-Petersen, Kalckar and Smith, 1955). This uridyl transferase catalyses a reaction between PGal and uridine triphosphate (URPPP) according to the equation:



$$\text{PGal} + \text{URPPP} \rightleftharpoons \text{PP} + \text{URPPGal}$$

This reaction is apparently predominant in plants (Neufeld *et al.*, 1957). Moreover, Isselbacher (1957) found the alternative transferase in rat liver. Hence, it is quite conceivable that the alternative transferase is present in small amounts in human liver and may increase in adults.

If congenital galactosaemia is a hereditary enzyme defect, one would expect to find partial defects in parents of galactosaemic children. The first approach to this problem was made by Holzel and Komrower in 1955. They used the galactose

* Most recently the transferase defect has been demonstrated in white blood cells (Weinberg, 1959) and in the lens epithelium (Lerman, 1959)

tolerance test as a criterion and found that either one or both the parents of galactosaemic children showed lower values in the tolerance test. The identification of one specific enzymic defect —the gal-1-P uridyl transferase defect—encouraged us to try to devise a specific test for the detection of heterozygous carriers.

In order to make a technique available for this purpose, it

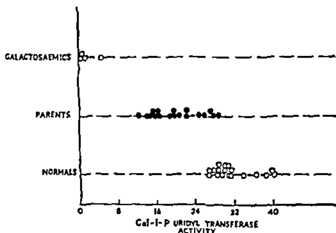


FIG. 2 The activity of gal-1-P uridyl transferase expressed as µl. O_2 consumed per hr. by 0 3 g. of red cells from three different classes of individuals. (i) Monogalactosaemico (mainly normal healthy individuals) (ii) Individuals suspected of being heterozygous with respect to the gene for the synthesis of the transferase since they have offspring with congenital galactosaemia (iii) Corresponding homozygous affected individuals, i e. galactosaemics (*Kirkman, 1959a. Courtesy of Ann. hum. Genet.*, Cambridge Univ. Press).

would be most desirable to have a method with a built-in control that ensures that one is measuring rates in the range of zero order kinetics. Kirkman used a very simple device, the old Barron-Warburg manometric determination of oxygen consumption in haemolysates (brought about by addition of methylene blue and metabolites). This method was so "tailored" as to fit the purpose of comparing transferase activities from normal and suspected galactosaemic carriers; it scores more than twenty points of measurements with high

accuracy. The details of the method have been described elsewhere (Kirkman, 1959*a*).

The manometric assay showed (Kirkman and Kalckar, 1958; Kirkman, 1959*a*) that about 80 per cent of the parents of the galactosaemic families which were surveyed had enzyme titres which were more than 2 standard deviations below the mean of non-galactosaemics (see Fig. 2). Kirkman is inclined to believe that if haemolysates from normals as well as haemolysates from brothers and sisters of suspected carriers were assayed, the manometric assay might identify practically all suspected carriers individually. This is the first time, to our knowledge, that a method for measuring a partial enzyme defect stands a chance of being used for purposes like consultation of parents in matters concerning eugenics and preventive medicine.* With reference to the latter aspect, from Kirkman's distribution curve (Fig. 2) it will

* A few methodological problems which need clarification are discussed briefly here. Kirkman emphasizes that the manometric method gives about 50 per cent of the maximum values which can be obtained by the two-step enzymic method designed for determining initial rates. This design was performed as follows (Kalckar, 1959). Small amounts of haemolysates (0·05 ml. of packed erythrocytes) were incubated at 37° for brief periods of time (e.g. 5 and 10 minutes) with relatively high concentrations of reactants (0·17 µmoles of URPPG and 0·85 µmoles of gal-1-P were used). If Tris buffer is used the rates of transferase in haemolysates from normal individuals (or, more correctly, homozygous non-galactosaemics) ranged from 3·5 to 5·8 µmoles/g cells/hr (Kalckar, 1959). In the presence of phosphate buffer (either at pH 7·3 or pH 8) the rates are consistently cut down to half. It is quite possible by the modified two-step enzymic method to find lowered rates in haemolysates from heterozygous carriers of galactosaemia, we found values as low as 1–1·5 µmoles (Kalckar, 1959). By the use of an almost identical set-up, Brettauer and co-workers (1959) recently obtained the following values: non-galactosaemic individuals, 4·8–8·1 µmoles; carriers of galactosaemia, 2·9–3·7 µmoles. These values are somewhat higher than ours. One may logically ask: why not use the enzymic technique just outlined as the method in population studies; especially since the manometric method is best reproduced in phosphate buffer at pH 7·3, conditions which permit only half of the maximum rates which can be found in Tris buffer?

The reason for our choice of the manometric method is that it shows the investigator whether he is measuring initial rates (zero order kinetics) or not, and this is the essential point in comparing enzyme rates from homozygotes and heterozygotes. The enzymic method as used by Brettauer and co-workers (1959), i.e. scoring one point, does not give such a guarantee and should therefore, in our opinion, not be used as the sole method "to detect parents who are likely to give birth to galactosaemics" (Brettauer *et al.*, 1959). The manometric method would be a much more reliable guide.

be evident that an appreciable fraction of carriers show enzyme titres below 50 per cent of the average of normal titres. This is of great importance in pregnancy, during which state the galactose tolerance is somewhat lowered for other reasons. If a pregnant mother, heterozygous with respect to the gene directing the synthesis of gal-1-P uridyl transferase, carries a homozygous galactosaemic foetus, excessive milk drinking during her pregnancy may expose the foetus to hazardous amounts of galactose. In a fraction of such cases an identification of the enzyme defect in the umbilical cord blood such as we described earlier (Anderson, Kalckar and Isselbacher, 1957) may therefore not be of much use with respect to dietary preventive measures. The preventive measures should have been used on the mother as well.

The incidence of hereditary galactosaemia is not known. We have had 35–40 cases under investigation, and in all the cases we have had the transferase was affected. In most of these cases we have secured that the other enzymes of the Leloir pathway are unaffected. Our clinical colleagues who have sent us samples can report on numerous cases of misdiagnosis of the disease (Bergren, Donnell and Kalckar, 1958).

It is too well known a fact to warrant any elaboration here that early diagnosis and institution of galactose-free diet is crucial. Otherwise, permanent cataract, intelligence defects and liver cirrhosis will ensue. The biochemical basis for the tissue damage is unknown as yet. The discovery by Schwarz and co-workers (1956) that gal-1-P accumulates in the erythrocytes of galactosaemic individuals put the spotlight on a most interesting problem: it has been found (Ginsburg and Neufeld, 1957; Sidbury, 1957) that gal-1-P inhibits phosphoglucomutase, i.e. the enzyme which catalyses the conversion of glucose-1-phosphate to glucose-6-phosphate. As emphasized, especially by Najjar (1959) it seems worth considering this inhibition as being responsible for the development of some of the tissue damage characteristic for galactosaemia.

In order to learn more about the genotypic and phenotypic

characteristics of hereditary blocks in gal-1-P uridyl transferase we have extended our search to micro-organisms. It has been known for some time, mainly through the work of the Lederbergs, that certain K_{12} strains of *Escherichia coli* are unable to metabolize galactose and are also unable to grow on galactose as the sole carbon source—so called gal-mutants (Morse, Lederberg and Lederberg, 1956). The many gal-mutants had so far only been characterized genotypically. The challenge was to try to characterize them phenotypically and more specifically with respect to the supposedly primary gene products, the enzymes.

Kurahashi in our group first undertook to apply the enzymic techniques used in the identification of the defect in hereditary galactosaemia to bacterial mutants (Kurahashi, 1957). It soon became apparent that there were at least four genotypically different mutants which were phenotypically identical, i.e. they all had a practically complete defect in transferase. Likewise, there were three different mutants [different "mutons" in same "cistron" (Benzer, 1957)] which phenotypically could be characterized as "galactokinase-less". Finally, there are various types of single mutants (Lederberg, 1958) "leaky" with respect to the two enzymes mentioned (i.e. greatly lowered activity as compared with the wild type) and more or less completely defective with respect to the 4-epimerase (Kalckar, Kurahashi and Jordan, 1959).

Characteristic for the genotypes which are unable to synthesize gal-1-P uridyl transferase is a phenomenon which we call "galactose-induced sensitivity" because the cells react like cells in an unbalanced state only if galactose permease and galactokinase are induced (Kurahashi, 1957; Kalckar, Kurahashi and Jordan, 1959; Yarmolinsky, Kalckar and Wiesmeyer, 1959). Under these conditions, gal-1-P accumulates in large amounts just as in the afflicted children mentioned above. In most of the *Esch. coli* strains with which we have been working (Lederberg's K_{12} galactose mutants) the abnormal state could be called "galactose-induced bacteriostasis" because the cells do not lyse and they remain viable

(Yarmolinksy, Kalckar and Wiesmeyer, 1959). The lack of lysis is borne out not only by the fact that turbidity and viable cell count remain constant but also by the fact that the medium does not contain more than traces of enzymes such as

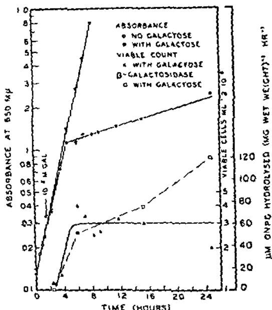


FIG. 3 Growth and division of *Esch. coli* K_{12} strain 3101 at 37° under aerobic conditions. Growth as well as viable counts plotted logarithmically. The activity of β-galactosidase (on a cellular basis) is plotted linearly and expressed as μmoles of *orthonitrophenyl galactoside hydrolysed* per hour per mg. toluenized cells (wet weight). The β-galactosidase activity of the cells which were grown in medium without galactose was too low to record on this graph (less than 1 μmol per mg. per hour) The β-galactosidase activity in the medium of non-toluenized cells grown with or without galactose is negligible (less than 1 μmol per mg. per hour).

β-galactosidase. This should be emphasized because Fukasawa and Nikaido (1959) have recently described galactose-negative mutants in which induction by galactose brings about lysis and death, unless the cells are grown in hypertonic medium in

which case cells more or less without a cell wall (spheroplasts, protoplasts) are formed. These "lytic" strains have a single defect in 4-epimerase (Kalckar, Kurahashi and Jordan, 1959).

In this case, "*galactose-induced bacteriolysis*", as well as in the case of "galactose-induced bacteriostasis", we have strongly selective conditions for two classes of mutants, either one of them "galactose-insensitives": (I) reversions, i.e. galactose-positives, and (II) additional mutants of gal

Table II

ACCUMULATION OF GAL-1-P AND UDPG LEVELS AS RELATED TO THE DEVELOPMENT OF "GALACTOSE-INDUCED BACTERIOSTASIS"

Hours		*μM/g. wet wt.*	
With 10^{-3}M gal	*Generation*	*Gal-1-P*	*UDPG*
0	1½	<0 2	
0	1½		0·19
3 1/10	2	0 4	
5½	5½	1 4	0 17
10	25½	4 5	0·22

permease or galactokinase, i.e. double mutants which are still galactose-negatives. The latter type are formed more frequently and we have identified one of them as a double mutant partly blocked in galactokinase on top of its transferase block.

The relevance of these studies on K_{12} mutants to the problem of human galactosaemia is obvious. We should be able to extract three types of information which may have more or less direct bearing on the human case. (I) Biochemical aberrations (accumulation of gal-1-P, possible changes in nucleotides, etc.). Some of the findings from our studies on one of the transferase-less strains in the absence and in the presence of galactose are summarized in Fig. 3 and in Table II

The static state coincided with a very appreciable accumulation of gal-1-P. The UDPGlucose levels were not affected in this strain. In other strains we have found lowering of uridine diphosphoglucose or of adenosine triphosphate during the static state. It is, however, doubtful that these differences are primary. (II) The nature of the genetic block. Do the transferase-less mutants represent "errors" in the gene which makes the enzyme, or aberrations in genes concerned with the production of enzyme? Kirkman has raised this problem in connexion with his studies on the hereditary glucose-6-phosphate dehydrogenase defect in Man (Kirkman, 1959*b*). In this case it is particularly relevant since Kirkman has been able to show that the "feeble enzyme" did not differ, with respect to a number of essential characteristics, from the normal enzyme. Since no qualitative or quantitative differences aside from catalytical activities have been detected, Kirkman feels that one should more seriously explore the possibility of a mutation in a gene concerned with production of the enzyme and not take it for granted that the defect is due to a "warped" enzyme with low turnover number. These considerations should not be left out, either, in a discussion of the gal-1-P uridyl transferase defect in hereditary galactosaemia. (III) The striking changes in population dynamics in cultures of transferase-less cells with galactose present invites an adventure into tissue and cell culture of cells from glactosaemics. It is possible to get human white blood cells to grow in tissue and cell cultures. Sensitive methods for galactose metabolism are being developed and it should, therefore, be possible to detect the appearance of very small clones of galactose-positive cells in a culture of galactose-negative cells from a galactosaemic individual. If there is also a selection in favour of the new clone in the presence of galactose, it should be possible to detect transformation of homozygous galactosaemic cells into heterozygous with relatively high transferase titres by the use of deoxyribonucleic acid preparations from "galactose-positive" individuals.

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DISCUSSION

Eagle: Prof. Kalckar, what is the basis for the positive inhibition caused by the galactose-sensitives?

Kalckar: This is a complicated story. Najjar (1959, *loc. cit.*) has emphasized the possibility that galactose-1-phosphate inhibits phosphoglucomutase. This was indeed found to be the case by work in his laboratory as well as in our own by Ginsburg and Neufeld (1957, *loc. cit.*) and Sidbury (1957, *loc. cit.*). Phosphoglucomutase catalyses the conversion of glucose-1-phosphate into glucose-6-phosphate. The bacterial mutase may also be inhibited by galactose-1-phosphate. In the galactose-sensitive bacteria there seems to be a correlation between the increase in generation time and the accumulation of galactose-1-phosphate although we cannot rule out the possibility that a threshold concentration is decisive. There are differences among the transferase-less galactose-sensitives. In some strains which we obtained from Tokyo, galactose brings about a dramatic lysis (Fukasawa and Nikaido, 1959, *loc. cit.*). In one of our static galactose-sensitive strains, uridine diphosphoglucose was decreased concomitant with stasis and accumulation of galactose-1-phosphate. However, in another static strain accumulation did not affect uridine diphosphoglucose levels. We may be able to differentiate therefore between various "mutons" of the same "cistron" but this is another matter. The cell pathology remains unexplained. We believe that the use of ^{14}C-labelled galactose should be of assistance for the clarification of this problem.

Pontecorvo: What is known about the distribution of the transferases and the other enzymes of this system in the various cells of the body tissues? The system is very active in the red cells, I gather?

Kalckar. Liver has, of course, the highest concentration. Curiously enough, galactokinase and UDPGal transferase are present only in small amounts in the lactating milk glands (Maxwell, E. S., Kalckar, H. M., and Burton, R. (1955). *Biochim. biophys. Acta*, 16, 444). The mammary gland which has plenty of epimerase seems to prefer to

make lactose from glucose or pyruvate rather than from galactose. Lactose synthesis in the mammary gland is quite complex as appears from the elegant studies by H. G. Wood (1959, *Josiah Macy Jr. Found. III Conf.*, Polysaccharide Biology, ed. Springer, G. F. Princeton). Likewise, brain which contains so much galactose has very little kinase and transferase activity. Epimerase is present in brain as well as in mammary glands (Maxwell, Kalckar and Burton, 1955, *loc. cit.*).

Childs: The inhibition of phosphoglucomutase which Prof Kalckar has mentioned could easily explain some of the symptoms in the galactosaemic infant. It has been shown that the administration of galactose to such babies is often accompanied by a serious reduction in the blood glucose of the infant. The reduction in glucose can easily be explained on this basis Perhaps there are other manifestations which could be explained similarly.

Kalckar: I agree with you, the mutase inhibition offers a basis. However, the rôle of the mutase inhibition in cell pathology has to be evaluated on a kinetic basis. We have tried in one case to see whether the elicitation of a sudden adrenaline hyperglycaemia would be affected by feeding galactose to an adult galactosaemic person. We did not find any decrease of this response. Despite the lack of positive evidence at the present time, I would not dismiss the possibility of the mutase inhibition being responsible for many of the disease symptoms of galactosaemia.

Harris: Prof Kalckar, you said that the brain galactosides were deposited exclusively after birth. Is that due to the fact that you get no epimerase in the foetus, or is there some other reason?

Kalckar: Dr. Kirkman and I have found, in unpublished studies, that in foetal liver of rats and guinea pigs there is very little uridinediphosphoglucose. This is also the case at birth However, ten days after birth it has risen to levels where it can be measured. The most beautiful studies stem from Brown and Zuelzer (Brown, A., and Zuelzer, W. W. (1958) *J. clin. Invest*, **37**, 332) They studied an enzyme which we described some years ago (Strominger, J. L., Maxwell, E. S, Axelrod, J., and Kalckar, H M (1957). *J. biol. Chem.*, **224**, 79), a specific dehydrogenase, uridinediphosphoglucose dehydrogenase, which catalyses the formation of uridine diphosphoglucuronic acid. It is one of the disharmonies of Nature that this enzyme which catalyses the formation of "active" glucuronic acid, and hence is so important for the detoxification of bilirubin formed from haemoglobin, does not appear in significant amount until about two weeks after birth. Therefore, in blood group incompatibilities giving rise to an increased production of bilirubin, the danger of free bilirubin being deposited in various tissues, including

brain, is high. Perhaps at some time in the future we should be able to induce this important dehydrogenase right after birth. The enzymes of galactose metabolism have been found to be present in embryonic rat liver (Isselbacher, 1957, *loc. cit.*).

Kalmus: A figure which struck me in your data was the amount of the blocked enzyme in the parents of affected children; this was about half the normal amount. Was there much variation and would you think that, if you find any measurement on heterozygotes to be half-way between the normal and the homozygous value, then you are measuring a process very near to the primary gene action?

Kalckar. The mean of enzyme level in the heterozygotes is about 60–63 per cent of that of normal according to Kirkman (1959*a*, *loc. cit.*). A significant proportion of heterozygotes have transferase levels of less than half of the mean of normal. Hence, if a heterozygous pregnant mother having low transferase values is drinking large amounts of milk a complication may arise if she also carries a foetus which is a homozygous galactosaemic. The chances of this foetus being exposed to galactose are undoubtedly high. As to the nature of the gene defect, I am forced at the present time to renounce any interpretation. The gene defect may not be directly connected with the assembling of the peptide forming the matrix for the transferase.

Cavalli-Sforza: How stable is the enzyme in the red cells? Can you hope to use stored red cells or not?

Kalckar: Yes, the haemolysates can be stored for several weeks. The diagnosis of homozygotes is "child's play". However, with respect to the heterozygotes you have to be much more careful. Yet it can be stored for a week without loss of enzyme activity (Kirkman, 1959*a*, *loc. cit.*).

Brenner: Is there a chance of finding out whether there is an altered protein?

Kalckar: At the present time work is mainly done on the microbial mutants. I am much more attracted to make an attempt with epimerase, which has a prosthetic group, a pyridine nucleotide. In *Esch. coli* K_{12} we have found an epimerase which has lowered specific activity. In one case the epimerase activity does not increase in the presence of galactose (Kalckar, H. M., and Kurahashi, K., unpublished observation) This may, of course, indicate a [illegible] a gene concerned with the regulation [illegible] rather than a mutation in the gene [illegible] synthesis.

CHOLINESTERASE TYPES

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THE enzyme cholinesterase is known under the various names of pseudocholinesterase (Mendel and Rudney, 1943), serum cholinesterase or s-type cholinesterase (Zeller and Bisegger, 1943), non-specific cholinesterase (Glick, 1945), butyrylcholinesterase (Sturge and Whittaker, 1950) and C-esterase (Augustinsson, 1958). The physiological function of cholinesterase is unknown but the enzyme is capable of destroying some drugs (Bovet-Nitti, 1949; Kalow, 1952). Cholinesterase is quite distinct in behaviour (Davies and Green, 1958) and occurrence (Ord and Thompson, 1950; Koelle, 1955) from acetylcholinesterase, an enzyme which is *important functionally in the nervous system* (*Wilson and* Cohen, 1953).

The present paper summarizes a series of studies performed over a period of several years, which have led us to conclude that there exist two slightly different inherited types of cholinesterase.

Reasons for starting the investigation

A widely used therapeutic measure is the application of electro-shock treatment to patients suffering from various forms of mental illness. During recent years it has become customary to apply a skeletal muscle relaxant prior to the administration of the shock. Such a relaxant will block the transmission of impulses between nerve and muscle so that the application of current to the skull, and thereby to the brain, will not result in visible convulsions. Usually, electro-shock treatment does not consist of a single session but rather of a series of treatments applied at intervals of days or weeks, so that a given patient may receive a muscle relaxant repeatedly. Thus, it was possible for Dr. D. R. Gunn several years ago to

observe that two of his patients showed a grossly prolonged response to the relaxant succinylcholine (suxamethonium) every time they received the drug. Succinylcholine (Bourne, Collier and Somers, 1952) is usually characterized by its short duration of action, i.e. the effects of a completely paralysing dose may not last longer than two or three minutes because of rapid destruction of the drug by cholinesterase. Dr. Gunn was therefore interested in measuring cholinesterase levels of those two patients.

With the intention of studying the relationship between thyroid function and cholinesterase, we utilized ultraviolet spectrophotometry to develop a new method for measuring levels of cholinesterase activity in human serum (Kalow and Lindsay, 1955). In order to check the validity of this new method, we were interested in measuring cholinesterase levels in a variety of patients, including Dr. Gunn's patients.

The new spectrophotometric method gave results on those two patients which were not in agreement with results produced by accepted standard methods. Furthermore, the spectrophotometer permitted the observation of progress curves of a reaction catalysed by cholinesterase (Kalow, Genest and Staron, 1956); these progress curves were of a peculiar shape when the sera of Dr. Gunn's patients served as the source of enzyme. These observations started the investigations which are to be reviewed here.

We were fortunate in having had the co-operation of the Staff of a Mental Institution, so that serum of these two patients was available again and again over the course of several years. A further 15 persons have been found whose serum cholinesterase behaved like that of the two prototypes but none of their sera could be investigated with regularity.

Biochemical investigations

1. Studies on normal and atypical cholinesterase of serum

The following section summarizes diverse biochemical comparisons, mostly between samples of serum of the two patients

described above, and the sera of two healthy research workers. The patients turned out to be atypical homozygotes and the research workers normal homozygotes in regard to cholinesterase. The enzyme in these samples of serum will be referred to as atypical and normal respectively.

The means employed for the measurements of enzyme activity were mostly ultraviolet spectrophotometry, as previously described (Kalow and Lindsay, 1955; Kalow and Genest, 1957). In several studies, gasometric measurements were performed with the Warburg apparatus. More recently, an automatic titrator was available which permitted the continuous measurement of acid produced during the hydrolysis of esters.

The action of the two types of cholinesterase upon various substrates. A comparative investigation of normal and atypical cholinesterase consisted in measuring the hydrolyses of 6 members of a homologous series of *n*-acylcholinesters, i.e. a series of esters including acetylcholine and heptanoylcholine (Davies, Marton and Kalow, 1959). The Michaelis constants of these esters and the atypical enzyme were from 1·5 to 6 times greater than the respective constants observed with the usual enzyme. In other words, there was a decreased apparent affinity of atypical esterase for each of these substrates. The maximum hydrolysis velocities of these cholinesters in sera with atypical esterase were between 2 and 5 times slower than in sera with an average level of normal esterase activity. Statistical tests permitted the conclusion that the ratios of the maximum activity of the two enzymes varied significantly from substrate to substrate. In most sera, butyrylcholine is hydrolysed noticeably faster than any other cholinester; this observation had given rise to the term butyrylcholinesterase (Sturge and Whittaker, 1950). In sera with atypical esterase, the rates of hydrolysis of butyrylcholine and pentanoylcholine by atypical esterase were nearly alike.

There is some chemical similarity between benzoylcholine and procaine (novocaine). Both these compounds are hydrolysed by cholinesterase (Kalow, 1952; Augustinsson, 1958).

Hydrolysis rates of these two compounds are characterized by "inhibition by excess of substrate"; i.e. the reaction rates become slower once an optimum concentration of substrate is exceeded (Kalow, Genest and Staron, 1956). For these substances, the optimum substrate concentrations were found to

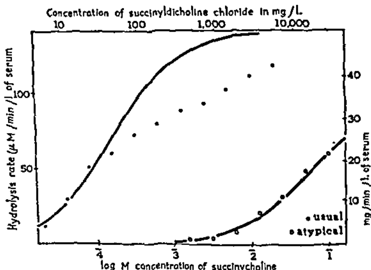


FIG. 1. Hydrolysis of succinylcholine by usual and atypical cholinesterase.

Both enzymes are capable of hydrolysing succinylcholine but they have grossly different affinities for the drug; this explains the prolonged effect of the drug in persons with atypical esterase. For the usual enzyme, the relation between concentration of drug and reaction rate grossly deviates from simple theory.

(From Kalow, 1959. Reproduced by permission of the Editors, *Anaesthesiology*.)

be higher with atypical than with normal esterase (Kalow, 1960).

The destruction of the muscle relaxant succinylcholine by the usual cholinesterase shows rather complicated reaction kinetics (Kalow, 1959). Nevertheless, the experimental data (Fig. 1) suggest that comparable affinities of succinylcholine for the usual cholinesterase are more than 100-fold greater than those for the atypical enzyme. The concentrations of

succinylcholine in serum after intravenous injection have not been experimentally determined but limiting values can be calculated; for instance, if a person has 3·5 l. of serum, and if 70 mg. of succinylcholine are injected, the serum levels cannot very well exceed a value of 20 mg./l. At that concentration, the drug can combine with normal esterase but not with atypical esterase (cf. Fig. 1). This seems to explain the prolonged response to succinylcholine of patients with atypical esterase (Kalow and Gunn, 1957).

Interaction with inhibitors. The substances best known for their inhibiting effect on cholinesterases are physostigmine (eserine), and organic phosphorus-containing compounds which are used as insecticides (Gilman and Koelle, 1949). However, the number of substances which are capable of blocking the cholinesterases is rather large, and includes numerous alkaloids and quaternary ammonium compounds (Augustinsson, 1948). It was therefore possible to find esterase inhibitors with potencies varying over a 20 million-fold range. The effects of such a series of inhibitors on the usual and atypical cholinesterase were compared. Results of this study suggested that inhibitors of cholinesterase could be divided into three different groups (Kalow and Davies, 1959*a*).

The first group consisted of phosphate inhibitors. The investigated members of this group were tetraethylpyrophosphate (TEPP) and isopropylfluorophosphate (DFP). These inhibitors blocked both the normal and the atypical esterase to the same extent, at least under the testing conditions employed. The rate of onset of inhibition may differ for the two types of enzyme but this needs further investigation.

The second group of inhibitors consisted of physostigmine and some derivatives of various synthetic alkaloids which are used in medicine as local anaesthetics and opium substitutes, and of such simple quaternary compounds as choline and tetramethyl ammonium (TMA). The normal and atypical enzyme seemed to be related by the exponential equation $I50_{atypical} = (I50_{usual})^{0.78}$; thereby I50 means the concentration of inhibitor causing 50 per cent inhibition under

standardized conditions. The equation suggests that the more potent an inhibitor the larger the difference between the inhibiting capacities for the normal and atypical enzymes. For instance, choline, procaine, neostigmine and its derivative RO2-0683 (Fig. 2), were four inhibitors in increasing order of potency; the ratios $I50_{atypical}/I50_{usual}$ with these inhibitors were 2, 14, 25, and 120, respectively.

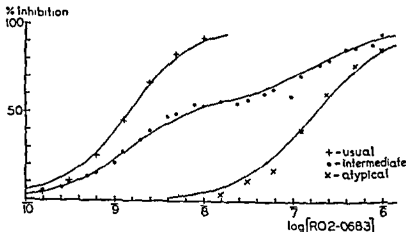


FIG. 2. Cholinesterase inhibition by the neostigmine analogue RO2-0683 in sera from three different persons.
Usual and atypical refer to the type of esterase in the respective sera. Intermediate indicates the serum of a person heterozygous in regard to esterase.
(From Kalow and Davies, 1959*a*. Reproduced by permission of the Editors, *Biochem. Pharmacol.*)

The third group consists of the muscle relaxants succinylcholine and decamethonium. The properties of succinylcholine as a substrate of pseudocholinesterase have been presented above (p. 42); under suitable experimental conditions the properties of succinylcholine as an inhibitor of the same enzyme can be tested. If succinylcholine and decamethonium had been acting like the second group of inhibitors, their inhibiting capacities towards atypical esterase should be roughly tenfold weaker than those towards the normal esterase. However, both agents were roughly 100-fold weaker in their effects towards the atypical enzyme than towards the

normal enzyme. Whatever the explanation for the special properties of succinylcholine, the results indicate that it has widely different affinities for the two enzymes whether it be tested as a substrate or as an inhibitor.

Some inhibitors differ not only in their strength of inhibition towards the two types of enzyme but also in their mode of inhibition (Kalow and Davies, 1959*a*). For instance, decamethonium, chlorpromazine and dibucaine were competitive inhibitors for the atypical enzyme but only partially competitive inhibitors for the usual cholinesterase. Furthermore, the slopes of log. concentration–inhibition curves of decamethonium appeared to be more shallow for the normal than for the atypical esterase.

The use of inhibitors gave the best means of distinguishing between the two types of esterase in routine experiments. A given concentration of inhibitor added to material containing esterase causes different intensities of inhibition, depending on the type of esterase present. It is obvious from the foregoing statements that a considerable number of inhibitors could be used for this purpose. However, dibucaine was usually chosen as the inhibitor because it is a readily available drug, it is stable, it blocks the esterases instantaneously and the slope of log. concentration–inhibition curves is sufficiently steep. Percentage inhibition in a system consisting of human serum diluted 1 : 100 with phosphate buffer at pH 7·4, with benzoylcholine 5×10^{-5}M as substrate and 10^{-5}M dibucaine as inhibitor, was termed dibucaine number (DN) (Kalow and Genest, 1957). Reference to the use of DN for the sorting of individual sera will be made below. Generally, DN >71 indicated normal esterase, DN <80 atypical eterase, and intermediate DN a mixture of enzymes.

Buffer ions and activators. Fig. 3 shows the influence of three buffers at various levels of pH upon the two enzymes. In phosphate buffer, at least one ionization constant (Laidler, 1958) must have a different value in the two enzyme-substrate complexes, since the pH optima are different. In Tris buffer and in glycine buffer, the pH optima of both enzymes

are probably alike. However, the activity of the normal enzyme is greater than that of the atypical enzyme.

As reported by Fraser (1956) and by Erdös and co-workers (1958), several alkaloids, amino acids and quaternary ammonium compounds are capable of enhancing the activity of serum cholinesterase under some experimental conditions.

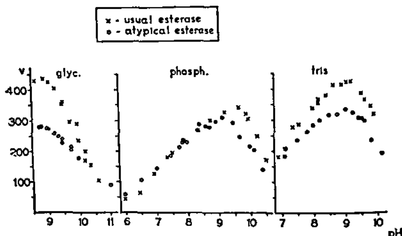


FIG. 3. The influence of buffers on the activities of usual and atypical cholinesterase of human serum

glyc. = glycine buffer; phosph. = phosphate buffer; tris = tris (hydroxymethyl) amino methane buffer The concentration of each buffer was M/15.
Abscissa = Observed, initial pH of reaction mixture.
Ordinate = Reaction velocities in arbitrary units measured by u.v. spectrophotometry with 5×10^{-5} M benzoylcholine as substrate. Sera were diluted so that their enzymic activities in phosphate buffer at pH 7·4 were alike.

We found activators of the normal enzyme always capable of activating the atypical esterase but not necessarily at the same concentration of activator and at the same pH. A detailed report is in preparation.

Electrophoretic studies. Several series of fractionation procedures failed to give any indication of a physical difference between normal and atypical esterase, i.e. ammonium sulphate precipitation, column chromatography with diethylaminoethyl (DEAE) cellulose (Peterson and Sober,

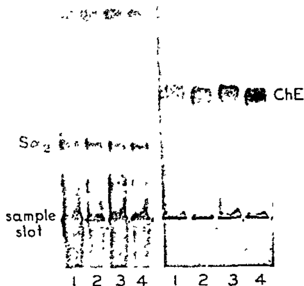


FIG. 4 Decreased electrophoretic mobility of normal cholinesterase due to selective attachment of decamethonium Starch gel electrophoresis (Smithies, 1959).

Of 4 samples of human serum, numbers 1 and 3 contain atypical, and numbers 2 and 4 normal cholinesterase. The figure shows two halves of the same starch plate, one half being stained for protein (left), the other for cholinesterase (right) The starch contained $1 \cdot 5 \times 10^{-5}$ M decamethonium. Time of electrophoresis was 40 hours with 3 5 v/cm The serum albumin has migrated off the gel.

facing page 47

1956), and paper electrophoresis. Using electrophoresis on starch gel (Smithies, 1959), Dr. O. Smithies was kind enough to compare in his laboratory the rates of migration of normal and atypical esterases; he found no difference. We confirmed this result in our laboratory and found identical migration rates for the two types of enzyme even if the experimental conditions were changed by using phosphate instead of borate buffer and by modifying the pH. A slight difference of migration rates of normal and atypical esterase could be achieved by adding the enzyme inhibitor decamethonium to the starch.

We first determined the dissociation constants of decamethonium for the two enzymes in borate buffer of pH 8·86. It was estimated that a concentration of $1{\cdot}5 \times 10^{-5}$M would cause roughly 90 per cent inhibition of the normal and about 10 per cent inhibition of the atypical esterase. Hence, $1{\cdot}5 \times 10^{-5}$M decamethonium was boiled with the starch in preparation for electrophoresis. To the bridge solution at the anode, 10^{-4}M decamethonium was added, so that the enzymes were constantly exposed to decamethonium during the electrophoresis. As expected, the normal enzyme was more strongly blocked than the atypical and its electrophoretic migration was therefore slightly delayed (Fig. 4). The two enzymes migrated at the same rate if the concentrations of decamethonium in gel and bridge solution were increased or decreased by a factor of ten. Since decamethonium is a reversible inhibitor with a competitive component of action, both enzymes could be stained in its presence.

The delay of migration of the normal enzyme (as shown in Fig. 4) was nearly 4 per cent. This may mean that four out of one hundred negative charges of the enzyme had been neutralized by decamethonium. Each molecule of decamethonium must be expected to neutralize two negative charges so that the observed delay of migration indicates the attachment of two decamethonium molecules per 100 charges. Longsworth (1941) has titrated an ovalbumin in 0·1 ionic strength and found four negative charges per molecular weight of 10,000 at pH 8. The molecular weight of human serum cholinesterase

is probably 300,000 (Surgenor and Ellis, 1954); the isoelectric points of the enzyme and ovalbumin seem to be in a similarly acid range. Thus, one might guess that there are 100–150 negative charges per cholinesterase molecule. Hence, the observed delay of migration of normal esterase is comparable with the assumption of an attachment of 2 or 3 molecules of decamethonium per molecule of enzyme.

Electrophoresis on starch also served for obtaining small quantities of normal and atypical esterase which were free of other recognizable constituents of serum. For this purpose, starch was prepared in the normal manner (Smithies, 1959) without decamethonium, and sera with either normal or atypical esterase were applied. After electrophoresis, the starch block was exposed to an esterase stain (Ravin, Tson and Seligman, 1951) for a few seconds so that one could just see the beginning of colour formation, sufficient to localize the enzymes. The coloured material is insoluble. The excess stain was washed off, small blocks of starch containing esterase were cut out, mashed up in saline and centrifuged. The supernatant contained the esterase activity, but so little total protein that there was no measurable u.v. absorption at 280 mμ. Both the normal and atypical esterase thus purified had the same susceptibilities to inhibition as they had in untreated serum.

2. Studies on heterozygotes in regard to esterase

As shown above, normal and atypical esterase differ greatly in some kinetic properties but their chemical structures are so similar that the two enzymes cannot be readily separated. It also has not been possible to obtain physical evidence for the presence of a mixture of normal and atypical esterase in the sera of heterozygotes. However, the cholinesterase activity of heterozygous sera is like that of an artificial mixture of esterases (Fig. 2). This has been recently illustrated with the aid of some enzyme inhibitors (Kalow and Davies, 1959*a*). The inhibitor which best discriminated between normal and atypical esterase was the neostigmine derivative R02-0683.

This agent was capable of selectively blocking the normal esterase in an artificial mixture of enzymes and has therefore been used to estimate the absolute activities of the two enzymes in heterozygous sera. Thereby, the impression gained by studying the distribution of DN (Kalow and Staron, 1957) has been confirmed. The proportion of the two enzymes varies from heterozygote to heterozygote. Possible mechanisms for the control of these proportions will be discussed below.

3. Cholinesterase from sources other than human serum

It may be noted in passing that DN in the sera of four Rhesus monkeys and of one horse were nearly the same as those of persons with normal esterase.

Some autopsy specimens of human brain and liver were obtained in order to check the properties of cholinesterase of tissue. DN could not be readily determined in the Warburg apparatus for various technical reasons. Thus an equivalent test, using 6×10^{-3}M benzoylcholine and 2×10^{-8}M R02-0683 with 50 min. preincubation, was devised. Under these conditions, the usual inhibition was 70–80 per cent in these two tissues. Among samples from 20 persons, liver tissue showed in two instances less than 20 per cent inhibition, while the corresponding brain tissues were strongly inhibited as usual. Unfortunately, blood specimens of the same subjects were not available. The investigations are being continued. There is evidence that serum cholinesterase originates in the liver (Vorhaus, Scudamore and Kark, 1950) so that an occurrence of atypical esterase in the liver would not be surprising.

Geneological and statistical studies

Relatives of persons known to possess atypical esterase either alone or in a mixture with normal esterase, were investigated (Kalow and Staron, 1957; Harris, 1958). Atypical esterase occurred in these relatives about ten times as frequently as in the general population (Kalow and Staron, 1957). It soon became obvious that the accumulation of

atypical esterase in families could not be a matter of "nurture". For instance, sibs had more often a similar type of esterase than spouses, although very few of the adult sibs lived together. Thus, we tested the hypothesis that there is a gene *A* for normal esterase and an allelic gene *a* for atypical esterase, and that each person has two genes for esterase. Then the three genotypes would be ***AA***, ***Aa***, and ***aa***, and the corresponding phenotypes would have normal esterase, the mixture, and atypical esterase, respectively. Among three such genotypes six combinations can occur (see Fig. 5). Four

A = normal gene; *a* = atypical gene.

*	*AA*	
A	*AA*	*AA*
A	*AA*	*AA*

*	*AA*	
A	*AA*	*AA*
a	*Aa*	*Aa*

*	*AA*	
a	*Aa*	*Aa*
a	*Aa*	*Aa*

*	*Aa*	
A	*AA*	*Aa*
a	*Aa*	*aa*

	Aa	
a	*Aa*	*aa*
a	*Aa*	*aa*

	aa	
a	*aa*	*aa*
a	*aa*	*aa*

* Observed combination

FIG 5. Possible combinations between genotypes. Assuming that there are two autosomal allelic genes without dominance for normal (*A*) and atypical (*a*) esterase, the asterisks indicate the combinations observed by Kalow and Staron (1957).

of these combinations have been observed and the proportion of phenotypes in the offspring was as expected from the genetical hypothesis.

Further support for the assumption of two allelic genes for esterase was obtained when the proportion of the three phenotypes was measured in some populations (Kalow and Gunn, 1959). *Among 2,017 healthy subjects were 74 persons* with a mixture of enzymes and one person with atypical esterase, the remaining 1,942 subjects having normal esterase. This distribution constitutes an agreement between observation and expectation ($\chi^2 = 0{\cdot}12$, $P > 0{\cdot}9$). The frequency of the atypical gene in the population, as calculated from these data, was $0{\cdot}019 \pm 0{\cdot}002$. A group of 2,500 mental

patients was statistically indistinguishable, in regard to esterase, from the healthy persons. Atypical esterase occurred with almost equal frequency in both sexes ($P > 0 \cdot 95$).

The investigated population consisted almost entirely of White persons living in Canada. An attempt was made to group the investigated population according to countries of origin, e.g. from Great Britain, Slavic countries, Middle Europe, France and Italy. There was no evidence of difference of gene frequency between these groups. A mixture of esterases has been observed in umbilical cord blood and in children of all ages but their numbers were too small to permit an estimate of frequencies of occurrence; among the adults there was no influence of age on the occurrence of heterozygosity.

Further statistical tests (Kalow and Gunn, 1959) were made to assess the possibility of a relation between atypical esterase and the following factors: body weight, religion, occupation and mental illness. None of these factors seemed to have anything to do with the frequency of occurrence of a typical esterase. The aspect of atypical esterase in mental illness has been particularly investigated because all the persons who had been first found to have atypical esterase were mentally ill. During the ensuing survey, there always remained some reason for suspecting a relation between esterase and illness, particularly between paranoia and atypical esterase. However, a special reinvestigation did not confirm the existence of such a connexion.

Lehmann and Ryan (1956) and Lehmann and Simmons (1958) have described the inheritance of "idiopathic low pseudocholinesterase levels". No experiments were done to assess the type of esterase Since sera containing atypical esterase have, on an average, decreased esterase activities, Lehmann's cases may well be like ours; the family trees suggest the presence of two autosomal allelic genes as in our data, and Lehmann's estimate of the frequency of homozygotes (Lehmann and Ryan, 1956) agrees with our calculations of gene frequency. In our experiments with benzoylcholine as substrate, atypical homozygotes usually show

atypical esterase in families could not be a matter of "nurture". For instance, sibs had more often a similar type of esterase than spouses, although very few of the adult sibs lived together. Thus, we tested the hypothesis that there is a gene *A* for normal esterase and an allelic gene *a* for atypical esterase, and that each person has two genes for esterase. Then the three genotypes would be *AA*, *Aa*, and *aa*, and the corresponding phenotypes would have normal esterase, the mixture, and atypical esterase, respectively. Among three such genotypes six combinations can occur (see Fig. 5). Four

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*	*AA*	
A	*AA*	*AA*
A	*AA*	*AA*

*	*AA*	
A	*AA*	*AA*
a	*Aa*	*Aa*

*	*AA*	
a	*Aa*	*Aa*
a	*Aa*	*Aa*

*	*Aa*	
A	*AA*	*Aa*
a	*Aa*	*aa*

	Aa	
a	*Aa*	*aa*
a	*Aa*	*aa*

	aa	
a	*aa*	*aa*
a	*aa*	*aa*

* Observed combination

FIG. 5. Possible combinations between genotypes.
Assuming that there are two autosomal allelic genes without dominance for normal (*A*) and atypical (*a*) esterase, the asterisks indicate the combinations observed by Kalow and Staron (1957).

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esterase is more active than atypical esterase, the normal esterase activity should predominate in an equimolar mixture of the two enzymes. Such a predominance of normal esterase activity in representative sera of heterozygotes has been shown with the aid of R02-0683, an inhibitor capable of selectively blocking the normal enzyme in the presence of atypical esterase (Kalow and Davies, 1959*a* and *b*). (4) The skew distribution of intermediate DN can be explained by the assumption that

	Esterase units	*Dibucaine numbers*
Father	215,207	65 8, 62 3
Mother	219,220	77 0, 78 8
Son	190,208	63 7, 67 9
Son, twin	126,131	41 3, 47 5
Son, twin	137,126	47 0, 50 8

FIG 7. Different intermediate DN within one family Cf. legend to Fig 6.
The twins have been shown to be identical.
(Data from Kalow and Staron, 1957)

heterozygous sera contain on the average normal and atypical esterase in equal molar concentrations but with unequal activities (Kalow and Staron, 1957).

Whether or not this assumption regarding the average heterozygote be true, there is evidence that the ratio of the two esterase activities tends to be constant for any given person but that this ratio varies between individual heterozygotes (Kalow and Genest, 1957). There is further evidence that this variation is subject to genetical control (Figs. 6 and 7). Unless there are numerous subtypes of cholinesterase with different turnover numbers, this observation reflects on the

quantitative aspects of enzyme formation: it suggests that the concentration of enzyme protein due to each gene is separately controlled. Fig. 8 shows esterase data in a family interpreted according to this assumption.

Neither the temporary reduction of cholinesterase levels due to liver dysfunction (Vorhaus, Scudamore and Kark,

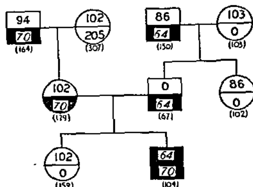


FIG. 8 Interpretation of family data, assuming genetical control of type and activity of cholinesterase. (Data from Kalow and Staron, 1957.)

A white field indicates a gene for normal esterase, a black field a gene for atypical esterase. The figures within these fields postulate units of esterase activity to be produced by each gene. The figures in brackets, underneath each symbol, give the observed esterase units, the average in a population with the normal type of esterase being 206 units.

While it is necessary to postulate a control of activity, the observed values do not conform rigidly.

1950) nor the induced esterase activity in tissue cultures (Burkhalter *et al.*, 1957) is evidence against the assumption of a genetical control of cholinesterase concentrations. Concentrations of esterases other than cholinesterase have been thought for many years to be inherited; thus some rabbits possess, and others lack, atropine esterase (Sawin and Glick, 1943; Ammon and Savelsberg, 1949). Gruneberg (1952) has reviewed the literature on differences of esterase levels in several strains of mice. Augustinsson and Olsson (1959) found

evidence for a genetical control of aromatic esterase activity in piglets. In each case, strict evidence for a control of concentrations was lacking; there might have been enzyme molecules with different turnover numbers. However, the total blood of a person contains nearly a kilogram of haemaglobin and perhaps 20 mg. of serum cholinesterase: the assumption is inescapable that the levels of proteins are under genetical control.

Summary

Two types of cholinesterase occur in human sera, and apparently in liver, which can be distinguished by their kinetic behaviour. The two esterases hydrolysed various substrates with different relative rates; they differed in their affinities for substrates and inhibitors, and in their susceptibilities to activators and buffers at various levels of pH. Small quantities of both enzymes were obtained in highly purified form whereby the characteristic features of the enzymes were unchanged. A different susceptibility to inhibition by decamethonium could be utilized to effect a slightly different electrophoretic mobility of the two enzymes in starch gel; no other physical separation was possible, indicating that differences must be confined to small areas at the active centres of the enzymes. The distribution and occurrence of atypical esterase suggests that there are two autosomal allelic genes without dominance for cholinesterase. Heterozygous persons possessed a mixture of the two enzymes in various proportions; also these proportions seemed to be genetically controlled. The frequency of the gene for atypical esterase was estimated as 0·02 in a White population.

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DISCUSSION

Brenner: These enzyme activities were expressed per unit volume of serum. Could one suppose that the control is not of the concentration of the enzyme in the serum but of the absolute amount in the whole organism? Presumably, cholinesterase is made by cells somewhere else in the body.

Kalow: There is some positive correlation between body weight and cholinesterase level in serum; hence the person-to-person variation of the absolute amounts of cholinesterase must be slightly larger than the variation of enzyme concentrations in serum

Brenner. One can imagine that the rate of synthesis of an enzyme can be controlled in various ways. Suppose that the enzyme is made by cells somewhere in the body, and that during development there is a cut-off point which fixes the size of population of these cells. If there is a time distribution of this process the number of cells could vary over quite a large range. This will then give variation of the level of the protein in the serum and such a case would be trivial from the point of view of looking for strictly genetically controlled mechanisms of control.

Smithies: Do I understand you, Dr. Brenner, to mean that the point at which the cut-off in the increase in the population of cells occurs is automatically determined when you have enough cells to produce the right amount of protein?

Brenner: No, what I am saying is that there could be many types of control mechanism. One, for example, may scan the amount of protein and, by feedback, regulate the rate of synthesis. Another type may be built into the structure of the protein itself, and I shall discuss this in my paper (this symposium, p 304). The third kind of mechanism, which could control the level of protein in a multicellular organism, is one which determines the number of cells that are producing this protein. Consider that there is a time during development at which the cells cease to multiply; if this is not fixed but variable then there might be a considerable variation in the size of the population.

Smithies: This, then, would not prevent it being necessary to have some genetic mechanism which determines when this cut-off occurs.

Brenner: Exactly, and microbial genetics provide the model for a good many ideas about this. In the multicellular organism, one must not only think of control mechanisms which operate through the protein itself. One must also consider mechanisms which operate on cell populations. These would be analogous, say, to the level of mutants in a bacterial population, rather than to anything which controls the rate of synthesis of protein molecules.

Kalow: Control of enzyme levels in the mammal is probably more complex than in single cell organisms. In the human body a nearly constant level of cholinesterase is maintained over a period of years; this level is subject to temporary fluctuations, for instance due to liver disturbance or due to such infections as the common cold. This means that the rates of enzyme production and of destruction must be balanced Only such a control gives sufficient flexibility to make a person regain the old enzyme level after the cold is over.

Ceppellini: Dr. Brenner, you mean that, for instance, hypoglobulinaemia can be due to a decrease in number of plasma cells and not to decreased synthesis of the globulin by single plasma cells?

Brenner. Yes. This is the possibility which must be kept in mind.

Ceppellini: What is the frequency of this low exceptional value?

Kalow We have made an estimate of gene frequency by investigating 2,000 healthy people, and obtained a value of about 0·02, which means that about 4 per cent of the population are heterozygotes and about 1 in 2,500 persons are atypical homozygotes. The best estimate was 1 in 2,800 (Kalow and Gunn, 1959, *loc. cit.*).

Harris: Dr. Kalow, I wonder whether from the experiments with different substrates and different inhibitors, and also the electrophoretic experiments, one could infer—assuming for example that the differences between the two enzymes are due to differences in amino acid sequence—what kind of amino acid one was dealing with. Most of your inhibitors are quaternary ammonium compounds and this, presumably, means a difference in the negative charge.

Kalow: The active centre of cholinesterases is thought to consist of an esteratic and of an anionic site. The former serves in the breaking of ester bonds, the latter orients the substrate molecules and thus influences the specificity of cholinesterases. There is no conclusive evidence for a difference between the esteratic sites of the two types of cholinesterase, since DFP inhibits both types to the same extent. The anionic sites of the two types of enzyme must be different, since tetramethylammonium (TMA) has different affinities for them. However, the binding of TMA to the anionic site involves not only electrostatic attraction but also van der Waals forces (Wilson, I. B. (1952). *J. biol. Chem.*, 197, 215); hence, I would hesitate to name specific amino acids which might be involved.

Ingram · The decamethonium that you used is a kind of detergent and the fact that it does not fit into your base-line relationship on the differential inhibition suggests to me that it is acting on a different part of the protein from the straight inhibitors of the amide type, in other words that you have altered the nature of the protein structure somewhere.

Kalow: This is an interesting way of looking at the data. I did not

show the detailed data on inhibition of cholinesterase by decamethonium. Curves relating log concentration of decamethonium to per cent inhibition are relatively shallow (Kalow and Davies, 1959*a*, *loc. cit.*). We have interpreted this as an indication that less than one molecule of decamethonium is necessary to block one active centre on the enzyme.

Ingram: With regard to the effect of phosphate buffers, it is well known that a triply charged phosphate will combine specifically with some proteins and alter their isoelectric point, whereas other monovalent buffers do not affect the protein in this way. In the presence of phosphate the optimum pH might well alter.

Kalow: By using a $1 \cdot 5 \times 10^{-5}$ M concentration of decamethonium, we expected roughly a 90 per cent inhibition of the normal enzyme and roughly a 10 per cent inhibition of the atypical enzyme. The choice of concentration of decamethonium was based on calculations of apparent affinities between the enzymes and the decamethonium. These affinities were calculated from inhibition data and Michaelis constants, considering that the enzyme inhibition by decamethonium was not completely competitive. Some error in choosing the concentration of decamethonium in the starch gel was immaterial because the concentration–inhibition curve was shallow; however, if we increased or decreased the selected concentration of decamethonium by a factor of ten, the two types of enzyme migrated at the same rate.

Pontecorvo: What is the distribution of cholinesterases in the various tissues?

Kalow: We have investigated some brain tissues and some liver tissues. These were autopsy specimens of persons whose serum, unfortunately, we could not investigate. Among the samples were two liver specimens which seemed to come from heterozygotes In the same persons, the brain esterase looked normal but this needs further investigation. Cholinesterase occurs in very many tissues: liver and pancreas are rich in esterases, also many parts of the brain; there are relatively large amounts in the hypothalamus and in the optic nerve, which may, however, not be associated with nervous structures. From histochemical studies we know that glial cells contain a relatively large amount. There is not much in skeletal muscle. Meissner's corpuscles of human skin have a rich supply of cholinesterase which suggests that it may have something to do with the functioning of the sensory nervous system; but various experimental approaches have failed to show a function of cholinesterase in the sensory system.

GENETICAL VARIATION AND SENSE PERCEPTION

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SENSE deprivation which is caused by mutant genes resulting in gross structural defect of a sense organ or the afferent nerve contributes but little to the problems of gene action, because the faulty chemistry involved in the maldevelopment of the sense organ and the chemistry of the receptor function are not identical; complete lack of the inner ear results in deafness, as does a badly developed or maintained organ of Corti in the mutants, kreisler and shaker, in mice (Grüneberg, 1952). Similarly in Man anophthalmos and degeneration of the optic nerve or the retina will result in blindness without shedding much light on the function of the retinal receptors. The developmental origin of some minor sense deficiencies—for instance, tone deafness or myopia—is also remote from the functioning of the acoustic or visual receptors but other minor deficiencies in sense perception may be more or less directly controlled by chemical genetical differences in a class of receptors, and may sometimes only be localized manifestations of more general metabolic peculiarities of the individual. There are indications that it may soon be possible to explain some of these defiencies by comparatively minor alterations of certain macromolecules which are part of the fine structure of the receptors concerned. Three such minor deficiencies in sense perception will be considered from this point of view, namely, congenital stationary night blindness, protanopia and taste blindness.

(1) Congenital stationary night blindness is inherited either as a dominant autosomal trait or as a sex-linked recessive (Bell, 1922) (Fig. 1). Night blind people do not see well in the dark and are very slow in dark adaptation, but show no impairment of daylight visual acuity. In some families the

affected members show, however, a slight defect of blue discrimination (Riddell, 1940), possibly indicating the involve-

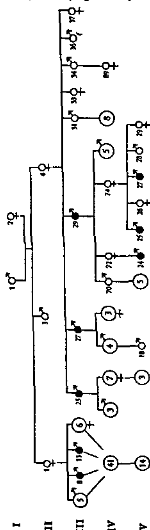


FIG. 1. Pedigree of hereditary stationary sex-linked night blindness. (From Bell, 1922.)

ment of a rod mechanism in blue discrimination (Konig, 1894; Willmer, 1949). Fig. 2 shows the rhodopsin-retinene-vitamin

A cycle in the rods. One may assume that the interference or block caused by night blindness would lie at the place indicated by the vertical arrows and would, in fact, make a conversion of vitamin A into retinene impossible or difficult. That lack of vitamin A can cause night blindness in a person or an experimental animal can be shown by feeding such an individual a diet lacking in vitamin A when, among other symptoms of avitaminosis, night blindness occurs. This kind of night blindness is reversible and quite different from the

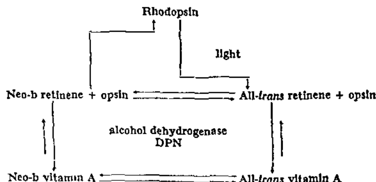


FIG. 2. Rhodopsin–retinene–vitamin A cycle. (From Wald, 1958.)

genetical stationary form which cannot be affected to any extent by massive doses of vitamin A.

The chemistry of the rhodopsin cycle has been and to some extent still is controversial (Dartnall, 1957). In particular, the importance of a bleaching product, "indicator yellow", has been stressed by a group of workers in Liverpool but denied by the Harvard School. The former assume that in rhodopsin a carbon nitrogen bond links the chromophore retinene to the protein (opsin), whereas the latter think that rhodopsin is retinene—sulphur—opsin. The difference between normal and night blind people might be a difference in an enzyme transforming the vitamin A into retinene or it might be the absence of an isomerase; alternatively it might be a difference in the opsin affecting its affinity to the retinene. Which of the

many retinene isomers are involved in light perception is not quite settled, as yet, but the suggestion by Wald (1958) that

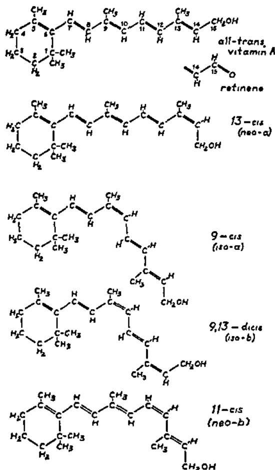


FIG. 3. Isomers of vitamin A and retinene (From Wald, 1958)

the neo-C (11 *cis*) isomer of the dark-adapted red rod is bleached through red/orange intermediates into the colourless all-*trans* retinene is at present widely accepted (Fig. 3). It is

possible that the rods of night blind people not only differ chemically from the rods of normal people but also have a defective fine structure in their outer segments. The complicated ultrastructure of the outer segments of rods and cones (Fig. 4) has only recently been discovered (Sjöstrand, 1953).

A possible arrangement of the visual pigments in the lamellar structures of the outer segments will be discussed after the consideration of the next condition. The impairment of blue discrimination in some night blind people perhaps indicates that their blue cones are affected by the same metabolic error, or a similar structural defect, as their rods, but it may alternatively indicate that blue sensation is not always or entirely mediated by the blue cones but can also result from a stimulation of rods.

(2) Protanopia, first recognizably described by Huddart (1777), and one of the four common sex-linked recessive forms of colour blindness, has recently been studied for the first time by objective methods (Rushton, 1958). Rushton analysed by an ingenious apparatus (Rushton *et al.*, 1955) the light reflected from the macula of normal and colour-defective people before and after prolonged exposure to intensive light of various wavelengths. It appears that the change of reflectancy, due to bleaching with strong light, of protanopes differs significantly from the change observed in normals, and Rushton concluded that a pigment was missing in their retina. The overall picture which emerges from these and similar findings agrees well with the conclusions drawn from the traditional subjective measurements and is as follows: in the cone receptors of normal Man, three pigments exist of which the blue-absorbing (cyanolabe) occurs in the so-called blue cones which do not concern us at present. Chlorolabe, with an absorption maximum in the green, occurs in the green cones, while the red-sensitive cones contain both chlorolabe and the red-absorbing erythrolabe. Protanopes appear to lack the doubly pigmented (red receptors) and deuteranopes the simpler green receptors. It is probable that the total number of cones

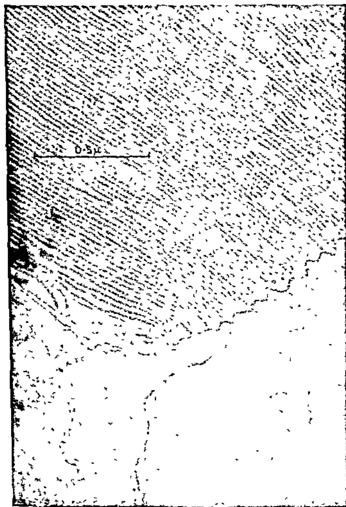


FIG. 4 Electron microphotograph of the outer segment of a frog's rod. (By courtesy of Mr. M S. Moody, Dept. of Anatomy, University College London)

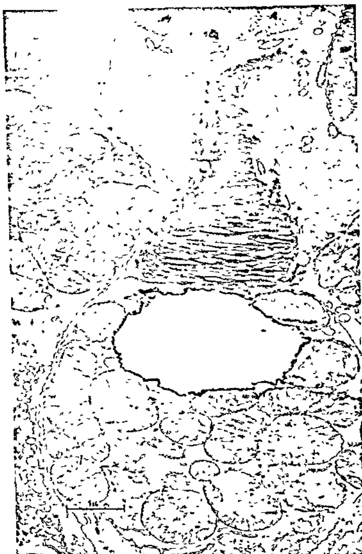


FIG. 6. Electron-microphotograph of the outer segment of a frog's cone. Beneath the double membrane structure an oil drop. (By courtesy of Mr. M. S. Moody, Dept. of Anatomy, University College London.)

in colour defectives is not reduced; no differences have been found so far in microscope slides from the retinae of colour blind and normal people; nor have the various types of cones been demonstrated. Whether or not electron microscopy will show any such differences remains to be seen. The observa-

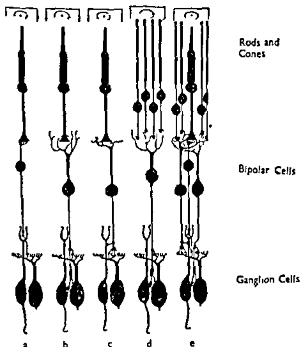


FIG. 5. The light receptors and their associated neurones in the retina (From Polyak, 1941)

tion of selective bleaching of retinal pigments in the living eye is difficult in such species as Man, who has no reflecting tapetum behind the retina, but in animals having such a structure, for instance crocodiles or cats, the phenomenon has been described long ago (Abelsdorff, 1896). Bleaching can also be observed in a preparation of the frog's retina where it has been shown (Denton and Wyllie, 1955) that the so-called

green rods and red rods are preferentially bleached by light of different wavelengths.

The question now arises how the differences in retinal bleaching of normal and protanopic subjects and in sensation can be correlated and interpreted in biochemical terms.

So far, to the author's knowledge, it has not been possible to demonstrate *in vitro* the existence of several cone pigments in any one vertebrate species, and similarly the physical fine structure of the light-sensitive outer segments of the cones has not, so far, given any indication of systematic variation; but inquiries into these two points seem now within reach. Fig. 5 shows the general lay-out of the retina according to Polyak (1941) and Fig. 6 is an electron micrograph of a total section and of the outer segment of a frog's cone. The relevant structure is again a finely folded membrane (double cell membrane)—perhaps not quite so regular as in the rod-forming flat layers vertical to the physiological light direction. Between these folds, molecules of photosensitive pigment are incorporated in an orientated manner: these are compounds of a retinene, perhaps again 11 *cis* (neo b) retinene (Wald, 1958) and proteins which are part of the cone structure different from the opsin of the rods and under control of the sex-linked genes. It need hardly be mentioned that, so far, nobody has investigated the cone pigments of colour blind people in solution, and it is possible that this would not be a very suitable approach anyway. As Wald (1958) has pointed out, "for a comparison of the correspondence between an observation spectra of the visual pigments and the spectral sensitivity of receptor vision, it is best to measure the observation spectra in the retina itself, or in rod or cone suspensions, since the spectra of the pigments in free solution differed from those *in situ*. We now recognize that in the visual receptors the pigments are orientated in highly organized structures that approximate the solid state."

Recently it has been shown (Hagins and Jennings, 1959) that the rhodopsin molecules in a rod have only one freedom of Brownian rotation and that their chromophores are most

likely arranged in a sort of monolayer between the lamellar structures similar to those in Fig. 6. This arrangement would make it possible that a single photon of green-blue light received and absorbed by one pigment molecule anywhere in the rod's outer segment might release a chemical alteration and produce a burst from the inner segment. The old idea that energy absorbed by one molecule might be transmitted to the inner segment by one or many molecules in free solution has thus to be abandoned.

In conclusion it might be said that, in the field of colour vision, genetical diversity, pigment chemistry, receptor structure and in fact colour sensation are approaching the molecular level from several directions and that results interesting for all concerned may soon be expected.

(3) The mechanics of the difference in sense perception which are now discussed have been explored only superficially but it may appear nearer to the theme of this symposium: it concerns the taste thresholds for phenylthiourea and other substances containing an —SCN group.

It has long been known (Fox, 1932; Harris and Kalmus, 1949) that about one-third of European populations (the "non-tasters") show a very low sensitivity to these substances while the "tasters" experience a bitter taste when very dilute solutions of such substances come in contact with their tongues. When thresholds are measured over a wide range of concentrations (Fig. 7), a two-peak frequency distribution appears, the antimode of which makes the classification into non-tasters and tasters of the majority of individuals possible. The testing of families and of sib pairs shows that non-tasting is an almost recessive autosomal trait. Non-tasters, as a group, are fully as sensitive as tasters to such bitter substances as picric acid or various alkaloids (Kalmus, 1958) (Fig. 8) and, therefore, the difference between the two phenotypes cannot be due to the lack of all bitter receptors in the non-tasters. Whether they lack a subclass of chemically specific receptors or whether all their bitter receptors are abnormal is unknown. The sensitivity of a receptor to any

tasting substance probably depends on a complex macromolecular mosaic on its excitatory surface, which may be compared to the complex mosaics on the surface of a red cell and which are responsible for its antigenic properties. (These are

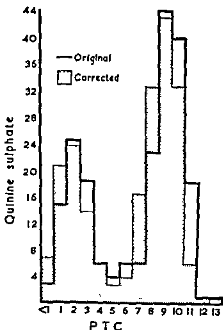


Fig. 7. Numbers of individual taste thresholds for phenylthiourea. The original bimodal distribution is slightly corrected for quinine sensitivity. (From Kalmus, 1958.)

dealt with by Prof. Morgan and Dr. Watkins in this symposium.) Comparatively slight differences in the arrangement of either receptor surface or compound may result in considerable differences in binding properties and the adsorption of larger molecules, especially if a large number of bonds is implicated (Beidler, 1958). In the —SCN compounds, for instance, the substitution of oxygen for sulphur changes the

bitterness as perceived by tasters into sweetness, and simultaneously raises the threshold of perception considerably

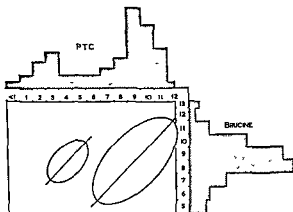


FIG 8. Bimodal distribution of PTC-thresholds and unimodal distribution of brucine thresholds for the same people. The ellipses indicate separate correlations of the two thresholds for PTC-tasters and non-tasters.

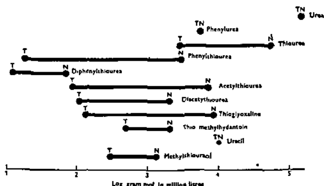


FIG. 9. Mean thresholds of various —SCN and —OCN substances for tasters (T) and non-tasters (TN). (From Harris and Kalmus, 1949)

(Fig. 9). Conversely, we may suspect that a slight difference in the surface mosaic of the receptors of a non-taster has a similar effect on the resulting sensation in so far as it does not

allow the adsorption of the tasting substances in the appropriate niche of the mosaic.

As yet, little is known about the physical details of the adsorption which brings any tasting substance into close proximity with the receptor molecules. Possibly the process of adduction, a sort of complex formation, provides a model (Weitkamp, 1958).

The "channels" in a crystal lattice of thiourea in methanol solution have a diameter of between 5·8 and 6·8 Å and it has been shown that limonene sulphides can just enter these channels whereas others—for instance, thiocineols—cannot. The concept of adduction of course closely resembles the old lock and key analogy of Ehrlich which—however disguised—is still a model of thought in many branches of immunology, biochemistry and toxicology.

The taster/non-taster difference in Man is probably localized in receptors contained in the vallate papillae of the tongue; but it may not be confined to these receptors and may, in fact, merely be a concomitant of a more fundamental metabolic difference in other tissues. Several —SCN substances are used in the treatment of toxic goitre and others occur in vegetables (Astwood, Greer and Ettlinger, 1949) and milk, and it has been shown that if ingested they can produce goitre in rabbits and in children (Clements and Wishart, 1956). It has been suggested that these substances produce a hypertrophy of the thyroid by interfering with the normal synthesis of thyroxine. In 1949, Harris, Kalmus and Trotter published some data on the taste thresholds of thyroid patients and suggested that non-tasters might be more prone to develop simple adenomatous goitre (nodular goitre) than non-tasters. More recently, Kitchin and co-workers (1959) have confirmed this observation, and have also found that toxic diffuse goitre is significantly more common among tasters. They suggested that the taster/non-taster genotypes are of importance in determining the type of thyroid disease which develops, but that they are not concerned in the factors which cause any such disease to develop. There are indications for the exist-

ence of considerable differences in taste sensitivity and overall resistance between individuals, strains and species of other mammals towards the —SCN substances, some of which are important rodicides (Richter and Dieke, 1947), but as yet we do not understand these differences.

In conclusion, concerning the use of subjective methods in the investigation of basically biochemical problems: while chemists are wont to crystallize and, if possible, to synthesize the substances which they study, and while biochemists somewhat more modestly want to watch reactions *in vitro*, it is quite unreasonable to expect that these traditional methods should always suffice to solve problems which are closely associated with cellular microstructure. In the investigation of such systems, methods must be used which do not destroy these structures and, in this respect, the methods of "exact subjectivism" are obviously most suitable. The relationship of this approach to the more commonly used objective methods is best illustrated by considering the study of sense organs as a study of instruments. Let us, for instance, compare the study of a colour blind eye to the investigation of a faulty colorimeter. While any single observer can test and compare several colorimeters, it is only the exceptional one-eye colour blind (Graham and Hsia, 1958) who can compare the sensations of two different eyes; but there is no reason why consistent and repeatable matches or measurements made under standardized conditions by several observers should not be validly compared. It would certainly be a mistake to expect that Rushton's methods will entirely supersede subjective measurements. Their importance lies in definitely localizing the defect of protanopes in the retina and in showing that objective changes and differences in retinal reflectancies are associated with the well established subjective changes and differences in sensation. After this demonstration, subjective methods of measuring colour perception which are greatly superior in ease of application and accuracy, will continue to be used with greater confidence and to better purpose. In general, studies of sensation will

probably continue to produce results relevant to the bio-chemistry of the sense receptors as well as to their genetical causation.

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DISCUSSION

Lederberg: Dr. Kalmus, would you comment on the Land phenomenon?

Kalmus: A fully coloured picture is produced by two colour components only when it is of some complexity; it depends on the interaction of retinal neighbouring areas and possibly on central integration; but also on the faculty of all cone types of responding to a wide range of wavelengths if the light is of sufficient intensity. The existence of separate cone mechanisms remained speculative for over 100 years until Rushton's demonstration. Subjectively two of the mechanisms can be shown quite easily. Any normal person can for instance be made a temporary protanope in half a minute by exposing his eyes to very strong red light. He will then be unable for a few seconds to read certain Ishihara charts If he bleaches only one of his eyes with red light his binocular colour match on the anomaloscope will contain a huge excess of red to match the standard yellow. Temporary deuteranopia can similarly be produced by green bleaching. Even more convincing is a demonstration of the separate various cone mechanisms in Stiles' adaptation experiments. If an intermittent blue light signal is matched against a permanently orange illuminated background the intensity of which can be increased, the colour of the flashing light at the threshold changes and a kink occurs in the curve.

Smithies: Dr. Kalmus, did you say there are some people colour blind in one eye who have normal vision in the other?

Kalmus: About sixty such people have so far been described. One such woman has helped to produce a film which I showed in 1958 at the Genetical Congress in Montreal, demonstrating what the world looks like to a colour blind person. By matching ordinary items of an interior scene, as seen with her defective eye, to similar items as seen with her normal eye she found that she had to remove all greens and reds so that only blue and yellow of various brightness and saturation remained in her visual world.

Smithies: Was this an acquired colour blindness in one eye?

Kalmus: It is the ordinary type of deuteranopia; but others as well as myself have tried in vain to get information about her family. In general, family histories of people colour blind in one eye found in the literature are badly reported but in some the condition seems to have occurred in both eyes in some relationship.

Kalckar: It would be interesting to see whether the Land phenomenon could be elicited in colour blind people.

Kalmus: I have produced the phenomenon in a deuteranopic colleague; but in my paper I purposely dealt with receptors especially

and talked less about colour vision in general. Of course, it should be realized that the simplicity of a sensation does not give any indication concerning the simplicity of the stimulus causing it. For instance, a red sensation may be mainly caused by one kind of receptor being stimulated. But it may also be caused by quite different situations. An equally uncomplicated yellow sensation is not caused by any kind of receptor—there are no yellow receptors—but requires at least the simultaneous stimulation of red and green receptors. White sensations require reactions from all three kinds of cones.

Pontecorvo: Are there any cases of mosaicism, where one eye is different from the other, i.e. one eye is a protanope and the other a deuteranope?

Kalmus: Such mosaicism might very well exist but cases reported in the older literature are rather dubious and the chances of such a person being detected in routine tests are slight.

Kalow: There are two physiologically occurring substances in the body that are bitter: earwax and bile. Are these appreciated normally by non-tasters?

Kalmus: I would assume that they are. When Harris and I tested some 40 or 50 bitter substances on people of known PTC threshold, two distinct kinds of result were obtained: either a clear-cut bimodal threshold distribution corresponding to the bimodal distribution of PTC-thresholds; or a simple unimodal threshold distribution hardly correlated to the PTC-thresholds. The first type of result was obtained only with substances containing at least one —SCN group; the second type of result was obtained with all sorts of bitter substances such as nitro compounds, alkaloids, extracts from citrus rind, etc. One may conclude from this that it is not the absence of all bitter receptors which makes a person a non-taster for PTC but some change in detail, either an absence of a subtype of bitter receptor or possibly a surface alteration in all bitter receptors.

Smithies: Quite by accident I recently came across a bitter substance which is present in a plant at a concentration tasted by some persons but not by others. I found a member of the Lactarius group of fungi which fitted the textbook description, except that the milk did not taste at all bitter to me as it should have done. Nor did it taste bitter to a friend who was with me; but to his wife it was extremely bitter. Three of their children were with us: to one it tasted bitter but to the other two it was quite bland.

Kalmus: From what you have said it appears that you are indeed dealing with human diversity and not with fungus genetics and this observation should certainly be followed up. It is quite possible that a —SCN substance may be responsible for the variable taste. As I

have mentioned, such substances occur in many higher plants such as cabbages and turnips.

Lederberg: There is a statement in several textbooks to the effect that the differential response to phenylthiourea depends on its being dissolved in saliva rather than water. Is that true?

Kalmus: Several years ago we did some rather disgusting experiments on this point, but I could never find anything positive. I think it is not true, but I am not sure.

THE GENETICS OF PRIMAQUINE SENSITIVITY OF THE ERYTHROCYTES

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Background

The last few years have witnessed the development of a unitary basis for a number of apparently unrelated drug-induced haemolytic anaemias. The principal impetus for this synthesis was given by a group at the University of Chicago, under the direction of Dr. A. S. Alving, who have studied comprehensively the haemolytic properties of antimalarial drugs as well as other substances. This work has been admirably reviewed and ably summarized by Beutler, himself one of the originators of the research (Beutler, 1959). Briefly, administration of the offending drug induces rapid haemolysis, followed by the expected events: anaemia, hyperbilirubinaemia and sometimes jaundice, falling haematocrit, and subsequently evidences of bone marrow regeneration. In many instances Heinz bodies are seen in the erythrocytes. After withdrawal of the inducing agent, and remission of the disease, the erythrocytes exhibit no apparent abnormality.

Taking their cue from many published reports of haemolytic anaemia due to primaquine, the Chicago group using prison volunteers to whom primaquine was administered, concluded that all persons were not equally susceptible to the haemolytic effects of that drug and that, in the populations they studied, only certain Negroes exhibited this propensity. The next steps involved description of the biochemical characteristics of the erythrocytes of susceptible persons, and invention of *in vitro* testing methods to facilitate the detection of "prima-

quine-sensitive" subjects. The biochemical characteristics found and studied are listed in Table I, which is based on a table presented in a paper by Alving and co-workers (1959). To this has been added the decrease in catalase described by Tarlov and Kellermeyer (1959). Of these, the levels of reduced glutathione (GSH), GSH instability, and lowered activity of glucose-6-phosphate dehydrogenase (G-6-PD) are critical, though their precise relationship to haemolysis remains obscure. Indeed the mechanism of the haemolysis of sensitive red cells is unknown.

Apart from the administration of primaquine to volunteers, four methods to detect sensitive persons were developed. The

Table I

A LIST OF SOME OF THE BIOCHEMICAL PROPERTIES OF PRIMAQUINE-SENSITIVE ERYTHROCYTES

Low reduced glutathione
Glutathione instability
Deficiency of activity of G-6-PD
Increased activity of glutathione reductase
Increased activity of aldolase
Decreased activity of catalase

first of these, quantitative assessment of Heinz bodies in the erythrocytes, was relatively inaccurate and is largely of historical interest (Beutler, Dern and Alving, 1955). The second, devised by Beutler, is the glutathione stability test (Beutler, Robson and Buttenweiser, 1957). Whole blood GSH is measured, after which the erythrocytes are incubated with acetylphenylhydrazine (APH), and finally the postincubation GSH is measured. In the non-sensitive individual there is relatively little change in GSH after incubation while, in the sensitive, there is a significant drop. The third method consists of accurate measurement of G-6-PD activity (Carson *et al.*, 1956). Several methods for the assay of this enzyme have been used by the various investigators, and though results are expressed in different units, all techniques are equally capable of detecting fully sensitive persons. Finally, an *in vitro* haemolysis method has been devised in which

^{51}Cr-tagged red cells from the test subject are injected into a known non-sensitive subject who then ingests primaquine, and the disappearance rate of the injected red cells can then be determined (Alving *et al.*, 1959). Obviously, different parameters of sensitivity are studied by these various tests and, as will become apparent, certain problems of discrimination are thereby created.

Genetical studies

A. GSH stability

The presence of a characteristic limited to certain individuals in a population and to one racial group suggests genetical origin of that characteristic. Beutler found about 9 per cent susceptibles or "reactors" among Negro males, and none among Caucasians (Beutler, 1959). These persons were selected as sensitive because the GSH of their erythrocytes dropped to levels of 20 mg./100 ml. of packed red cells (RBC) or less after incubation with APH. Beutler's curve was completely bimodal, revealing a gap between the values of 20 and 40 mg./100 ml. of packed RBC. In a Baltimore survey involving 144 males, and using similar criteria, 14 per cent of sensitive persons were found (Childs *et al.*, 1958). In the same survey 184 females were tested. Of these, only 2 per cent fell in the category of 20 mg. or less, while 5 per cent of the women had values falling between 20 and 40 mg. per 100 ml. of packed RBC, i.e. in the zone unoccupied by the values from any male. This difference was much more striking in the distributions of values obtained from members of families ascertained by the presence of a reactor. The distribution of male family members was exactly similar to that of the survey group, but that of values from females was continuous. These data thus suggest that in a population of American Negroes there are two kinds of male and three kinds of female. The two kinds of male have been designated reactors and normals; the three kinds of female are called reactors, intermediates and normals.

In the Baltimore study, 16 families including 30 sibships were examined. The characteristic appeared in one form or other in three generations in several of the families, suggesting dominant inheritance. When both forms of the characteristic (reactor and intermediate) were considered there were equal numbers of males and females, but it is noteworthy that there were no males in the intermediate range, and that the female reactors numbered less than half the male reactors. Assuming that the characteristic is in fact inherited, this distribution among the members of the families suggested that in the males there was true dominance while, among females, reactors were homozygotes, intermediates were heterozygotes, and values falling in the normal range were found among homozygous normals. Examination of GSH stability tests of persons in relation to their position in the families, however, introduced a new factor. Now it was apparent that relatively few of the male reactors appeared as fathers of persons who showed the characteristic, and that apparent male to male transmission appeared only once. The usual picture was one of an intermediate female who produced reactor sons or intermediate daughters. All the sons of reactor mothers were reactors, and their daughters were usually intermediates. It was also apparent in the pedigree analysis that in some families, reactor sons and intermediate daughters were progeny of parents neither of whom showed any degree of the trait; i.e. some women presumed to be heterozygotes had postincubation GSH values which fell into the normal range, indicating that some females possessing the gene failed to show any manifestation. This, however, was never found among any of the males. Analysis of these pedigrees together with the differences in expression between males and females in this study led to the conclusion that the gene was located in the X chromosome, that the reactor males were hemizygotes possessing the gene, that the reactor females were homozygotes, and that heterozygotes showed GSH values which were either intermediate or in the normal range. The data fit this hypothesis very well, the only major inconsistency

variance with the others (Alving *et al.*, 1959). Though the hypothesis of sex-linkage was substantiated, this method differed from the GSH stability test in discriminating sensitive females. Males whose erythrocyte GSH was unstable also showed *in vivo* haemolysis under the conditions of the test, while those with normal GSH stability tests showed no *in vivo* haemolysis. On the other hand, among females there were some inconsistencies. Some women whose GSH stability values were intermediate failed to show haemolysis, while a larger number, whose GSH values were normal, did show haemolysis. Though no data are given concerning a correlation between *in vivo* haemolysis and G-6-PD assays, Alving states in his paper that haemolysis was seen in females whose enzyme activities were *near* normal, but none was found in those whose enzyme activity was actually within the normal range. It is emphasized that the *in vivo* haemolysis method gives no intermediate values; the intensity and rate of haemolysis is just as great in a female with "near normal" enzyme activity as in a male with severe enzyme deficiency. Thus, for detection of susceptible persons of all types the *in vivo* haemolysis test is better than the GSH stability test, and better than most of the early enzyme assays, but for discrimination between heterozygotes and the two kinds of homozygote the other tests are superior.

Favism

In its symptomatology favism is similar to drug-induced haemolytic anaemia. Some substance in the fava bean (*Vicia faba*) is apparently responsible since the disease may follow ingestion of the bean (Luisada, 1941; Sansone, Piga and Segni, 1958). The condition has been known and studied for many years among the ethnic groups inhabiting the Mediterranean basin, and its familial aspects have been noted. It is certainly more frequent among males, but few genetical studies have been carried out, perhaps because of the inconsistency of the family data. Notwithstanding, a study

has been presented by Sartori based upon 215 cases of favism observed in Sardinia, and without benefit of biochemical methods (Sartori, 1957). Sartori's conclusion was that only persons possessing a constitutional factor were susceptible and that the mode of inheritance was as a recessive characteristic.

Because of the similarities between drug-induced haemolytic anaemia and favism, the discovery of the biochemical aspects of primaquine-sensitive erythrocytes has prompted an examination of patients who have experienced favism, and their families, from the new point of view. Wherever favism and drug-induced anaemia or primaquine sensitivity as defined by GSH stability tests or G-6-PD assay have been compared, they have been found to be very similar. Indeed Larizza and his associates have applied the definitive test and have shown that patients who have had favism are sensitive to primaquine, by administering standard doses of that drug to such patients and then tabulating day by day the fall in haematocrit, the rise in reticulocytes and the progressive, though mild, elevation in the serum bilirubin (Larizza *et al.*, 1958).

Several papers have offered data on the genetics of this condition using the GSH stability test and assay of G-6-PD (Zinkham, Lenhard and Childs, 1958; Gross, Hurwitz and Marks, 1958; Szeinberg and Sheba, 1958; Ventura, Grignani and Brunetti, 1958). Among these are the studies of Ventura and co-workers in Sardinia in which the enzyme assay was used. In this study based upon 16 families the familiar classes of males and females were found, and again there was no male to male transmission, suggesting agreement in this disease also with the sex-linkage hypothesis. It was of interest that their method of enzyme assay gave values of zero activity in their patients who had experienced favism. Examination of the data of Gross, Hurwitz and Marks, reveals that the G-6-PD values of Negro males which fell into the reactor range were higher than those of the Italians and Greeks whose values also designated them as reactors (2·74 ±

1·32 versus 0·37 ± 0·55, $P = < 0{\cdot}001$). Similar values are given by Szeinberg and Sheba who, using a different method for G-6-PD estimation, studied the characteristic among Jews (Szeinberg and Sheba, 1958). For 15 sensitive males the mean activity was zero. These observations have been confirmed by Zinkham who points out that this may be a consistent difference between the Negroes and Whites (Zinkham, 1959). If so, this may be a reflection of genetical as well as biochemical differences.

Studies of the enzyme itself

A genetically determined loss in activity of an enzyme poses a very pointed question of the precise nature of gene action. This challenge in the case of primaquine sensitivity has been met by Kirkman who has investigated the enzyme to determine whether its loss of activity might be due *to*: a molecular change involving the catalytic site, quantitative lack of enzyme molecules, enzyme inhibition or lack of an activator (Kirkman, 1959*a* and *b*). Kirkman has achieved an 80-fold purification of enzyme from a normal individual and from a primaquine-sensitive person who had 10 per cent residual activity. These two preparations he has compared in a number of ways, in most of which they behaved identically. Michaelis constants were the same for both triphosphopyridine nucleotide (TPN) and G-6-P, as well as for 2-deoxyglucose-6-phosphate. pH optima were also similar. When both preparations were passed through an anion exchange column (diethylamino ethyl cellulose), both showed identical movement and a single peak. Other parameters were also studied and no differences were found. Kirkman therefore believes that his studies offer no evidence that the mutant has produced a new molecular species, and that it is likely that there is neither an inhibitor nor lack of an activator. Though Kirkman is tentative in his conclusions it is perhaps permissible at this stage to guess that there is a quantitative lack rather than a qualitative difference in the enzyme in the primaquine-sensitive individual.

Congenital non-spherocytic haemolytic anaemia

Congenital non-spherocytic haemolytic anaemia is a relatively uncommon disorder in which there is no morphological abnormality of the erythrocytes. It is sometimes familial, the lifespan of the red cells is reduced, reticulocytosis is the rule and there may be crises of anaemia and jaundice. Biochemical characteristics of the erythrocytes of a few such patients have been studied using the methods which have been applied to the primaquine-sensitive erythrocytes, and in some cases similar aberrations have been found (Newton and Bass, 1958; Zinkham and Lenhard, 1959; Shahidi and Diamond, 1959). It should be emphasized, however, that not all such patients show these abnormalities (Zinkham and Lenhard, 1959). While Newton's case was of Italian extraction, those reported by Zinkham were of European descent, an ethnic group among whom the characteristic and presumably the gene have been shown to be very rare. Though exacerbations of the haemolysis can be excited by ingestion of appropriate drugs or by infections in some such patients, the clinical features of the disease distinguish it altogether from the "primaquine-sensitive" subject. The question, therefore, is raised whether a different gene is operative or perhaps the same mutant in a different genetical background.

Incidence and distribution

Drug-induced haemolytic anaemia is so widespread throughout the world that opportunity has been afforded workers in many areas to study its incidence in a variety of populations. Incidence values are given in Table II, which is based upon a table to be found in Beutler's review (Beutler, 1959). Only those reports have been included in which the incidence among males is given, since from such figures gene frequencies are readily calculated. Problems of discrimination among females make this difficult so these have been left out. Added to it are data taken from our own study of the frequency of the characteristic among Greeks living in Baltimore (Childs and Zinkham, 1959). All these persons were either born in

Greece or if born in the United States are the offspring of immigrant parents. The method used for detection of sensitive persons was the GSH stability test. It is of interest that among 75 persons studied all those showing any degree of abnormal manifestation came from the Island of Rhodes as opposed to other parts of Greece or of Turkey. Inhabitants of Rhodes, as well as certain of the other islands, have long

Table II

INCIDENCE OF PRIMAQUINE SENSITIVITY AMONG MALES IN VARIOUS POPULATIONS

Method	*Group*	*Number tested*	*% Reactors*	*Reference*
GSH stability	Negro	34	8 8	Beutler (1959)
	Oriental	51	2 0	Beutler (1959)
	European White	30	0 0	Beutler (1959)
GSH stability	Negro	144	14 6	Childs *et al.* (1958)
GSH stability	Ashkenazic Jews	203	0·0	
	Sephardic Jews	267	11 2	Szeinberg and Sheba (1958)
In vivo haemolysis	Negro	72	11·1	Alving *et al.* (1959)
GSH stability	Sardinian	61	13 1	
	Italian (Liguria)	100	0·0	Sansone, Segni and DeCecco (1958)
GSH stability	Greek	21	10·0	Childs and Zinkham (1959)

known favism, while reports of the disease on the mainland are infrequent (Luisada, 1941). Added also are data of Sansone on Sardinians (Sansone, Segni and DeCecco, 1958). In Sardinia, of course, the incidence of favism is very high (Sansone, Piga and Segni, 1958).

A glance at Table II reveals that the characteristic is not only geographically disseminated, but also that wherever it has been found, it is rather common. The only exceptions to this rule are the rare patients with congenital non-spherocytic anaemia who are of European ancestry. Thus, it is apparent that gene frequencies among the affected populations are remarkably high.

The future

Clearly much of this exciting and absorbing story remains to be told. We know very little about the relation of the glutathione deficiency and enzymic abnormality to haemolysis. Nor are all the other biochemical changes fully understood, though Alving has suggested that some of these may be secondary to the G-6-PD defect and compensatory (Alving *et al.*, 1959).

From the genetical viewpoint, one of the principal problems is that of discriminating the female heterozygote from the two homozygotes, in other words the reactors, intermediates, and normals A qualitative difference in the enzyme protein might have resolved this question, but failing that, we must fall back upon attempts to refine existing techniques. For instance, it is possible that the use of agents other than APH or other incubation times might sharpen the focus of the GSH stability test. A refinement which gives much promise is being worked out by Zinkham (Zinkham and Lenhard, 1959), based on a method of Glock and McLean (1953). In this test, generation of reduced TPN by oxidation of 6-phosphogluconate (6-PG) is measured, as well as that generated by oxidation of G-6-P and 6-PG together. By subtracting the former value from the latter, a more accurate estimate of the activity of G-6-PD is obtained. This test appears to introduce a trimodality into distributions of values obtained from Negro females. In some instances persons whose GSH stability tests are normal have been shown by the above method to have intermediate enzyme values.

Nothing is known of allelism, or of the possibility of genes at different loci, though it is reasonable to suppose that these might exist. The differences in G-6-PD activity in affected Negro males as opposed to affected White males is strongly suggestive. So also is the finding of continual haemolysis together with markedly reduced enzyme activity in the patients with non-spherocytic haemolytic anaemia; but equally, these three syndromes might be the result of insertion of a mutant into a genotype not prepared to modify its effects.

The frequency of the gene determining this characteristic is extraordinarily high, reaching in Iraqui Jews the figure of about 20 per cent (Szeinberg and Sheba, 1958). When considered together with its geographical dissemination, such frequencies should make the characteristic a valuable anthropological tool. The question also arises of a possible selective advantage which might account for such numbers of a gene whose only known effect is a deleterious one.

In summary, a new genetical entity has been described; one which should be as useful as the haemoglobin variants, blood groups and serum protein differences, in its contribution to our understanding of the genetics of human populations. It is possible, even probable, that in common with these other genetically determined differences "primaquine sensitivity" will be shown to be a heterogenous bag associated with a constellation of genes. It is important, therefore, that whatever studies are carried out in efforts to describe more completely this interesting condition, observations should be made on family groups rather than exclusively upon populations of unrelated individuals.

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DISCUSSION

Siniscalco: Some data have been collected very recently by our group on the incidence of G-6-PD deficiency in Sardinia and its relation to favism. These data are preliminary and incomplete but some of our findings are worth mentioning even at this stage for the benefit of future researches. One of the main aims of the investigation, which was carried out jointly with Dr. A. Motulsky (Seattle), Prof. U. Carcassi (Siena), Prof. B Latte (Nuoro, Sardinia) and my collaborators, Drs. M. Adinolfi and L. Bernini, was to test Dr. Motulsky's hypothesis that malaria could have been a selective agent for the maintenance of this trait in human populations. Other projects were the study, in non-hospital samples, of the relationships between the enzymic deficiency and favism, and the collection of more family data. Enzyme activity was determined in the field by Motulsky's test which is very rapid and reliable for screening purposes, as was later proved on re-examining a large number of samples (among which were all those found to be enzyme-deficient, and their relatives) by a spectrophotometric method similar to that described here by Dr. Childs.

In order to satisfy the main aim of this investigation we started by screening random samples of males in three villages located in the lowlands of the eastern coast of Sardinia (Galtelli, Orosei and Siniscola), known to have been highly malaric, and in three non-malaric villages (Desulo, Fonni and Tonara) on the mountains of the Gennargentu (altitude 3,000 feet).* These villages included those

* Assuming that the sex-linked hypothesis is correct and that the populations are in equilibrium, the proportion of males deficient in G-6-PD is a good estimate of the gene frequency.

which have been already investigated by Prof. Ceppellini (1959, *Ciba Found. Symp. Medical Biology and Etruscan Origins*, p. 177, London: Churchill) with regard to the incidence of thalassaemia and the distribution of blood groups. He found that the villages of the two areas could be considered as typically Sardinian in their blood

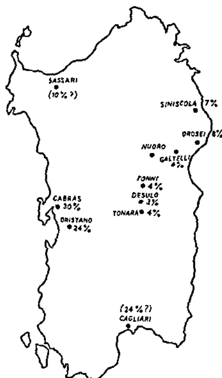


FIG. 1 (Siniscalco) Distribution of G-6-PD deficiency in Sardinia.

group distribution, but that the frequency of thalassaemia was much higher in the lowlands (about 18 per cent) than in the mountain region (about 3–4 per cent). I shall not discuss the question of whether this evidence is enough to permit the assumption that malaria might have played a selective rôle in the maintenance of the thalassaemic gene because, at least in the present case, even if the distribution of G-6-PD deficiency seems to indicate such a trend, *the differences* are so slight that the evidence is still inconclusive.

The following findings were of interest: (1) The incidence of G-6-PD deficiency as measured by the screening test was significantly different on the east and west coasts and lowest in the central mountain villages where malaria had not been present previously (Fig. 1). (2) About 20 per cent of the samples collected from the lowland villages of the eastern side (G-6-PD deficient individuals and their relatives) were found to have a weak haptoglobin pattern. Since previous data collected in a neighbouring Sardinian area did not reveal depressed haptoglobin and since thalassaemia (at least in the Po valley) also does not depress haptoglobin levels, we probably can rule out genetically determined ahaptoglobinaemia as well as phenotypic ahaptoglobinaemia due to haemolysis from thalassaemia as a cause of the observed depression of haptoglobin levels. Since subjects with low haptoglobin levels tended to have low or intermediate enzyme activity, on quantitative enzyme assay, it is likely that the weak haptoglobin patterns reflect the presence of active subclinical haemolysis in deficient subjects. Products of blossoming fava bean fields (or other toxic agents?) probably represent the haemolytic agent

The qualitative screening test discriminates perfectly between enzyme-deficient and normal male individuals in the absence of haemolysis but does not detect enzyme-deficient persons during or after haemolysis when many young cells which still contain significant enzyme activity are present in the blood stream. Consequently, individuals with subclinical haemolysis due to G-6-PD deficiency may be classified as normal by the screening test during the haemolytic episode. It is therefore possible that the actual incidence of G-6-PD deficiency on the east coast may be significantly higher than that detected by us during the fava season; this is indicated also by failure to demonstrate enzyme deficiency in a few patients with a positive history of favism.

Penrose: Dr. Motulsky has studied the distribution of these characters, and has told me that there are data on the age groups of these various types of people in Sardinia, which is rather important.

Siniscalco: Yes, this data was collected by Sansone and co-workers, and indicated that in childhood there is about 10 per cent of gene frequency in males and the ratio of affected males to affected females is 10 to 1, whereas among adults it is 1 : 2. So there seems to be a modification with age. Perhaps if one looks at this from the point of view of haemolysis it may prove to be a question of whether there is a difference in the distribution of mild or strong cases of favism with age.

Kalmus: Does sensitization play any part in causing favism? Perhaps it could account for these changes in frequency with age.

Siniscalco: Yes, sensitization is involved; I don't know what Dr. Childs thinks, but the school of Prof. Marcolongo in Italy thinks so. One feels that this enzymic abnormality is necessary in order to get favism, but it is not enough, and perhaps something else is required for the manifestation of the disease

Childs: It makes some difference whether or not the beans are cooked. The cooked bean is far less injurious than the raw bean. Furthermore, it would appear that severe cases are sometimes seen in people who simply passed by a field where the flowers are in bloom.

Ceppellini: That is a story!

Kalmus: As regards the question of possible sensitization, in some malarial countries quinine has been used for a considerable time, occasionally causing haemolysis and the lethal blackwater fever. If quinine sensitivity is hereditary, selection should act against it in malarial regions and reduce its frequency. Acquired sensitization, however, would not be selected against and would thus maintain its frequency of occurrence.

Siniscalco: Dr. Motulsky has evidence from the Belgian Congo of a very low frequency in reactors (about 2–3 per cent) in high mountains and a very high frequency (about 30 per cent) in the lowlands. But the mountain population, the Vatuzzi, originated in another part of Africa.

Harris: Dr. Childs, is there any independent genetical data about non-spherocytic haemolytic anaemia?

Childs Four or five families have been reported (Zinkham and Lenhard, 1959, *loc. cit.*) in which the family studies follow the usual patterns, i.e. an intermediate mother, one or two affected sons, and so on. As Zinkham has pointed out, however, other cases are apparently not genetically determined and do not exhibit these chemical abnormalities.

Harris. Have they chronic haemolytic anaemia?

Childs: Not necessarily. One can be sure about some of these people only by doing survival studies. In others one can determine the presence of the disease in the individual by episodes of jaundice and so on, and by reticulocytosis.

Neel: I believe that in the discussion after Dr. Motulsky's paper at the recent meetings in Atlantic City, Dr. Alving pointed out some previously unreported data to the effect that about 10 per cent of Eskimos also showed the primaquine sensitivity trait.

Ceppellini: That was drift!

Kalmus: But this is what you would expect. They have not been selected against: they did not take quinine.

Childs: None of the antimalarial drugs are likely to cause death; i.e. one can give primaquine in the usual doses day after day for a

year. This has been done by Alving and his group, and in a fairly short time the haematocrit rises to normal levels, so that it is unlikely that the administration of antimalarials is a very great evil as a selective agent.

Ingram: Does quinine give this effect?

Childs: Apparently very little.

Lederberg: Have you any more to say about the mechanism of haemolysis? You said that oxidants had something to do with it.

Childs: The reasoning that led to the discovery of abnormality of glutathione and G-6-PD was that the injurious agents might, after they got into the cell, become oxidized and act as oxidizing agents. In an attempt to find something which might protect against oxidation, glutathione was tried. But very little is known about the mechanism of haemolysis.

Smithies. Dr. Childs, you say that prolonged administration of the antimalarials does not appear to produce any lasting serious effect and so makes it difficult to see what selective forces are involved in changing the gene frequencies, but the population in which these tests were made is well nourished. In a population where there is, for example, a possibility of iron deficiency there might be some selection against individuals in whom haemolysis was constantly occurring

Penrose: The real problem here is that practically all the individuals with this enzyme deficiency are at a disadvantage. They get favism with the bean and they get haemolysis if they take an antimalarial drug. How is it possible for such a gene to be so prevalent? I think that is the problem which many of us have in mind and which Dr. Motulsky examined very critically. If the gene gives protection against malaria, a high incidence in Sardinia would be suggestive but, as Dr. Childs says, it would be very important to make more surveys in other districts which have been malarious I don't know what the details are of other European or Mediterranean countries where there is a lot of malaria, but I understand that in the eastern Mediterranean there are quite a number of communities which have a high incidence of this enzyme deficiency, and this rather suggests that there might be a real advantage there If it is true that favism is less severe in the female than in the male, the female heterozygotes have some advantage.

Ceppellini: I have already spoken of the so-called Mediterranean disorder, which implies thalassaemia, favism and also a haemolytic disease which is probably congenital, non-spherocytic anaemia. These have the same distribution pattern. As regards an advantage, if we accept malaria—and I don't say that we must accept malaria—it is reasonable to think that any disturbance of red cells will be

advantageous, because probably there is normally a perfect symbiosis between the parasite and the host; therefore in thalassaemia and similar disorders the defects can all have the same effect because they disturb the symbiosis.

Lederberg: Dr. Childs, could you comment on Marks' report that the juvenile erythrocytes are normal and the older ones are not?

Childs: Marks has shown that in the young cells from a sensitive person the enzyme activity is high. In reticulocytes, for example, the enzyme assay is pretty close to normal. So the defect is one which is found to a greater degree in the older cells and might in a sense be regarded as premature ageing.

Lederberg: Then does an age-sensitive "mutant enzyme" replace a normal component, or are both present in normal cells, the mutant cells having only the age-sensitive component? How do *in vitro* preparations of the enzyme behave with respect to loss of activity on ageing? One should study preparations from young cells whose activity would not already have been lost.

Childs: This has not been studied so far as I know. I think it would be worth investigating it in individuals after a haemolytic episode where there was a large amount of reticulocytes, or in patients who have haemolytic anaemia and who are always in a phase of reticulocytosis.

Ceppellini: A problem which is certainly not new in this case, but which can be raised for any sex-linked defect is why is the hemizygous normal male better off than the heterozygous female? If we assume that the mutant gene is a complete amorph then there should be some kind of dosage effect. In fact, the heterozygous female has less activity than the male. I think this is the real problem of alleles.

Childs: This has not been done in the formal sense. Until there are more data and a more precise method, it is very hard to comment.

Cavalli-Sforza: Dr. Siniscalco, on the question of distribution of gene frequency in connexion with malaria: in the eastern area of Sardinia where you said that the frequency of primaquine sensitivity is small, I understand there is a very high incidence of thalassaemia and I wonder whether the incidence of primaquine sensitivity might be negatively correlated with the frequency of thalassaemia, in those areas of Sardinia where malaria has been prevalent.

Siniscalco: So far, there are no reliable population data on thalassaemia for the west side of Sardinia. This is a very important problem, and we are going to study the distribution of thalassaemia and also the distribution of the enzyme deficiency in thalassaemic populations already studied, such as in the district of Ferrara.

Ceppellini: Marcolongo speaks of D.E.M. (haematological Mediterranean disorders) which include the thalassaemias, favism, some

non-immunological jaundices of the newborn, etc. (1953, Anemie Emolitiche, Ed. Pensiero Scientifico, Roma). All these syndromes seem to have the same geographical pattern of distribution (i.e. Sardinia, Sicily, Calabria, Greek coast). If we accept malaria as the selective agent responsible for this distribution (I clearly state that the malaria hypothesis has not yet been proved) it is reasonable to think that many different congenital defects, which disturb the biochemistry of the red cell, are likely to be favoured in a malarial area because they interfere with the perfect symbiosis which has been reached, through evolution, between the parasite and the host.

CHEMICAL AND GENETICAL UNITS OF THE HAEMOGLOBIN MOLECULE

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THE studies to be covered in the present discussion began as an inquiry into what appeared to be incompatibility between the structural characteristics of the inherited variants of human adult haemoglobin and the haemoglobin composition of heterozygous red cells. X-ray diffraction studies (Perutz, Liquori and Eirich, 1951) and chemical analyses (Ingram, 1957, 1958) had indicated that haemoglobins A (normal adult) and S (sickle cell) are symmetrical molecules; i.e. the protein molecules corresponding to the "wild-type" gene and its mutant allele are both composed of identical halves. Alleles controlling the synthesis of units no larger than the respective half-molecules would suffice to determine the structure of each molecule. However, consideration of the haemoglobin components of heterozygous cells led to an apparent paradox (Itano, 1957). Each red cell in sickle-cell trait contains both haemoglobins A and S. If these cells synthesize half-molecules which pair at random to form molecules of haemoglobin, a hybrid molecule composed of a half-molecule of haemoglobin A and a half-molecule of haemoglobin S would be one of the products. The same inference holds if the two types of polypeptide chains (Rhinesmith, Schroeder and Pauling, 1957) of each half-molecule are synthesized independently. Since only one of the pairs of chains of haemoglobin S is known to be abnormal (Ingram, 1957), the composition of the hybrid molecule would be the same whether the primary products of biosynthesis in a heterozygous cell are half-molecules or

chains (Fig. 1). The absence of hybrid molecules in heterozygous cells (Table I) implies either that identical half-molecules

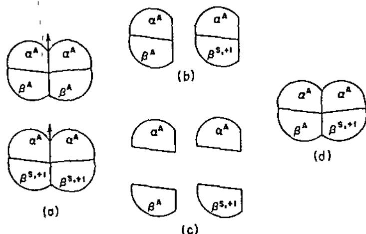


FIG. 1. Schematic two-dimensional representations of (a) haemoglobins A (above) and S, (b) half-molecules of haemoglobins A and S, (c) polypeptide chains of each half-molecule, and (d) a hypothetical hybrid molecule. The hybrid molecule would be the same whether the primary units of biosynthesis are half-molecules or polypeptide chains. Hybrids of this type are not present in heterozygous cells and have not been produced synthetically.

Table I

COMPONENTS OF HETEROZYGOUS CELLS

Condition	*Haemoglobin components*	
	Present	*Absent*
Sickle-cell trait	$\alpha_2^A\beta_2^A$, $[\alpha_2^A\beta_2^S]^{+2}$	$[\alpha_2^A\beta^A\beta^S]^{+1}$
HbC trait	$\alpha_2^A\beta_2^A$, $[\alpha_2^A\beta_2^C]^{+4}$	$[\alpha_2^A\beta^A\beta^C]^{+2}$
S—C disease	$[\alpha_2^A\beta_2^S]^{+2}$, $[\alpha_2^A\beta_2^C]^{+}$	$[\alpha_2^A\beta^S\beta^C]^{+3}$

or chains are paired during biosynthesis or that unlike units are physically incompatible. The report by Field and O'Brien

(1955), that carbonmonoxyhaemoglobin (HbCO)A dissociates reversibly in acid into subunits suggested an *in vitro* test of these possibilities.

Nomenclature

The two types of polypeptide chains of haemoglobin A are distinguished by their N-terminal amino acid sequences, val-leu for the α chain and val-his-leu for the β chain (Rhinesmith, Schroeder and Martin, 1958). The defective chains of the abnormal haemoglobins had not been identified when the results of our dissociation-association experiments were reported. Nevertheless, a symbolic representation was necessary for description and interpretation of our results. Haemoglobins S and C are abnormal in the same chain (Hunt and Ingram, 1958), and the abnormal chains of haemoglobins S and C and their normal analogue in haemoglobin A were designated s, c, and a, respectively (Itano and Singer, 1958). The other type of chain, which appears to be the same in all three haemoglobins, was designated u. The nomenclature was extended to include differences in chemical state of the haem group and in net charges (Singer and Itano, 1959). Haemoglobin I is apparently abnormal in the chain which is normal in haemoglobins S and C (Murayama and Ingram, 1959; Ingram, 1959; Itano and Robinson, 1959); therefore its abnormal chain, designated i, is an analogue of the u chain.

Vinograd, Hutchinson and Schroeder (1959) and Ingram (1959) have recently found that the defect of haemoglobin S is in the β chain. We can therefore replace our nomenclature with chain designations that include this new information. Table II relates the new designations to the ones that we have previously used. Formulae of molecules and subunits of the inherited forms of haemoglobin used in our studies are shown in Table III. The abnormalities in net charge near neutral pH are included in order to facilitate interpretation of electrophoretic data. Knowledge of the total charge on a subunit or molecule is not necessary for this purpose. Abnormalities of +1 for β^S and +2 for β^C arise from the substitution of valine

and lysine, respectively, for a glutamic acid of β^{A} (Ingram, 1957, 1958; Hunt and Ingram, 1958). The amino-acid sub-

Table II

NOMENCLATURE OF POLYPEPTIDE CHAINS OF HUMAN ADULT HAEMOGLOBIN

	Symbol		*Occurrence*
	*New**	*Old†*	
Normal chains	α^{A}	u	A, S, C
	β^{A}	a	A, I
Abnormal chains	α^{I}	i	I
	β^{S}	s	S
	β^{C}	c	C

* The superscript terminology for the α and β chains was adopted by mutual agreement with W. A. Schroeder and J. Vinograd.
† This terminology was used in our previous papers (Itano and Singer, 1958, Singer and Itano, 1959, Itano and Robinson, 1959)

Table III

CHAIN NOMENCLATURE OF HAEMOGLOBIN

Hb	*Chain*		*Molecule*	*1/2-Mol*	*Subunit*	
	α	β	$\alpha_2\beta_2$	$\alpha\beta$	α_2	β_2
A	α^{A}	β^{A}	$\alpha_2^{A}\beta_2^{A}$	$\alpha^{A}\beta^{A}$	α_2^{A}	β_2^{A}
S	α^{A}	$\beta^{S,+1}$	$[\alpha_2^{A}\beta_2^{S}]^{+2}$	$[\alpha^{A}\beta^{S}]^{+1}$	α_2^{A}	$\beta_2^{S\ +2}$
C	α^{A}	$\beta^{C,+2}$	$[\alpha_2^{A}\beta_2^{C}]^{+4}$	$[\alpha^{A}\beta^{C}]^{+2}$	α_2^{A}	$\beta_2^{C,+4}$
I	$\alpha^{I,-2}$	β^{A}	$[\alpha_2^{I}\beta_2^{A}]^{-4}$	$[\alpha^{I}\beta^{A}]^{2}$	$\alpha_2^{I\ -4}$	β_2^{A}

stitution that gives rise to the abnormality of -2 in the charge of α^{I} is not known. We are primarily concerned here

with relationships among the polypeptide chains of inherited variants of human haemoglobin. The specific chemical differences that characterize these chains are discussed elsewhere in this volume (Hunt and Ingram, 1959).

Acid Dissociation and Recombination of Haemoglobin

The starting point of the experiments to be described was the observation by Field and O'Brien (1955) that HbCO A dissociates reversibly below pH 6 into units of about half the size of the parent molecule. This result suggested to us the possibility that if HbCO S and C also dissociate reversibly in acid, acidification of a mixture of two different forms followed by neutralization might result in the formation of hybrid molecules composed of a half-molecule from each of the original components. Determinations of the sedimentation constants of acidified solutions and of acidified and neutralized solutions of HbCO A, S and C, disclosed that the three forms are equally susceptible to reversible dissociation in acid (Itano and Singer, 1958). The series of experiments to be described revealed that neutralization of acidified mixtures of haemoglobin does indeed result in exchange of subunits of the original components by a specific mechanism of recombination. However, the mode of dissociation and recombination is such that formation of hybrid molecules that contain two different inherited analogues of the same type of chain does not occur.

Recombination of HbCO A, S and C (Itano and Singer, 1958).

Mixtures composed of the three possible pairs of components, carbonmonoxyhaemoglobins (HbCO) A, S and C, were acidified to pH 4·7 and 4·3 and neutralized after 20–60 minutes. The mixtures thus treated yielded the same electrophoretic composition as corresponding untreated mixtures and mixtures of separately acidified and neutralized components. A true hybrid molecule (Fig. 1 and Table I) with a net charge

and mobility between those of the original components would have appeared as a new electrophoretic component. Two possible mechanisms are consistent with these results: (1) HbCO dissociates symmetrically into half-molecules (Table III); however, unlike half-molecules are physically incompatible, and only recombination of like half-molecules to restore the original components occurs. (2) HbCO A, S and C, dissociate asymmetrically into unlike subunits, each of which is a symmetrical pair of chains. For example, a mixture of HbCO A and S would dissociate into $\alpha_2^A + \beta_2^A$ and α_2^A and $\beta_2^{S,+2}$, respectively. Exchange of α_2^A subunits derived from the two haemoglobins would not be detectable electrophoretically. This mechanism can be tested by labelling one of the original components and analysing for exchange of the label after recombination.

Recombination of mixtures of HbCO and Hb+: Haem-labelling experiments (Singer and Itano, 1959):

A specific charge-label can be placed on each haem group by oxidation to ferrihaem. The charge on each haem of ferrihaemoglobin (Hb^+) is greater than that of HbCO by one unit in acidic buffers, and the cationic mobility of Hb^+ is correspondingly greater than that of HbCO (Itano and Robinson, 1958). Acidification and neutralization of either HbCO or Hb^+ separately did not result in any new electrophoretic components. Recombination of mixtures of HbCO and Hb^+ of the same haemoglobin resulted in a third component of intermediate mobility. Hb^+ has four more positive charges per molecule than HbCO. The intermediate component migrates with a mobility which corresponds to a molecule with two more charges than HbCO and apparently is a haemoglobin molecule with two CO- and two ferrihaems. Thus each product of acid dissociation carries two haems, and no detectable dissociation into single chains occurs under our experimental conditions. However, this result does not distinguish between symmetrical and asymmetrical dissociation. The six possible mixtures of HbCO and Hb^+ of haemoglobins A, S and C, were

recombined after acid dissociation and analysed electrophoretically. Fig. 2 shows electrophoretic patterns of untreated and

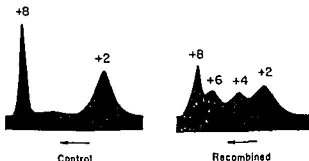


FIG 2. Moving-boundary electrophoretic patterns of control and recombined mixtures of HbCO S (+2) and Hb+ C (+8) The arrows indicate the direction of migration, and the numbers represent excess in net charge per molecule over that of HbCO A. (From Singer and Itano, 1959 Reproduced by permission of the Editors, *Proc. Nat. Acad Sci., Wash.*)

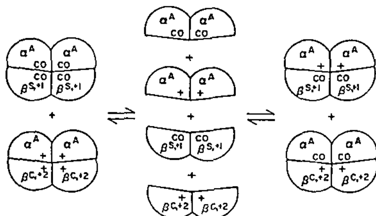


FIG. 3. Asymmetrical dissociation and recombination of HbCO S and Hb+ C. Dissociation of original components (left) to subunits (centre) followed by recombination into four species (left and right) with charges of +2, +4, +6 and +8, accounts for the electrophoretic pattern of Fig. 2.

recombined mixtures of HbCO S and Hb+ C. The abnormality in charge of HbCO S is +2. The abnormality in charge of

Hb+ C with respect to that of that of HbCO A is +8, the sum of +4 for its identical pair of amino acid substitutions and +4 for the four ferrihaems. Recombination of these components resulted in two new components with mobilities corresponding to charges of +4 and +6. As shown in Fig. 3 this result is in accord with asymmetrical dissociation and recombination. Each of the two new products of recombination contains two ferrihaems in addition to the respective charge abnormalities of +2 and +4 for haemoglobins S and C. Results with the other five mixtures were also consistent with asymmetrical recombination but not with symmetrical recombination.

Recombination of [2-^{14}C]glycine-labelled HbCO A with unlabelled HbCO S and C (Singer and Itano, 1959).

HbCO A from patients who had received injections of [2-^{14}C]glycine in connexion with other investigations (Nathan

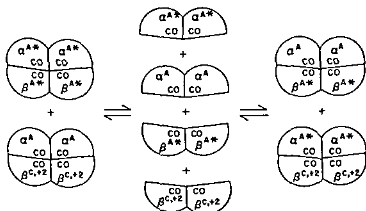


FIG. 4. Asymmetrical dissociation and recombination of [2-^{14}C]glycine-labelled HbCO A and unlabelled HbCO C. Partial transfer of ^{14}C activity from HbCO A to HbCO C without change in electrophoretic components is consistent with asymmetrical recombination to form half-labelled molecules (right) in addition to the original components (left).

and Berlin, 1959) and had incorporated ^{14}C activity into their haemoglobin was mixed with unlabelled HbCO S and C.

Acidified and neutralized mixtures yielded the same electrophoretic components as untreated mixtures. However, when the components were isolated by starch block electrophoresis and counted, transfer of some of the ^{14}C activity from HbCO A to HbCO S or C was observed in recombined mixtures. Fig. 4 shows how asymmetrical recombination results in partial transfer of radioactivity from labelled HbCO A to unlabelled HbCO C without formation of new electrophoretic species. Recombination of $\alpha_2^{A*}\beta_2^{A*}$ and $[\alpha_2^{A}\beta_1^{C}]^{+4}$ results in a mixture of $\alpha_2^{A}\beta_2^{A*}$, $[\alpha_2^{A*}\beta_2^{C}]^{+4}$ and the original components. When equimolar mixtures of labelled and unlabelled molecules recombine at random, one-quarter of the radioactivity is transferred from HbCO A to HbCO C.

***Recombination of HbCO I with HbCO S and C** (Itano and* Robinson, 1959). **The use of recombination experiments to identify the altered chain of abnormal haemoglobins.**

Since homogeneous haemoglobin I was not available, a mixture of haemoglobins A and I from a patient studied by



FIG. 5 Moving boundary electrophoretic patterns of control and recombined mixtures of HbCO A, HbCO S, and HbCO I. The recombined mixture (right) shows a new component migrating between HbCO A and HbCO I, increase in HbCO A, and decrease in HbCO S and HbCO I. (From Itano and Robinson, 1959.)

I. R. Schwartz and co-workers (1957) was used. Recombination of HbCO and HbCO I did not alter the composition of the mixture and suggested that one of the pairs of chains is the *same in both molecules.* As shown in Fig. 5, recombination by

acidification and neutralization of this mixture with HbCO S resulted in the appearance of a new component migrating between HbCO A and HbCO I, increase in the proportion of HbCO A, and decrease in the proportions of HbCO S and HbCO I. Recombination of HbCO A with HbCO S or with HbCO I does not alter the electrophoretic composition of a mixture. Therefore, the observed changes must be due to

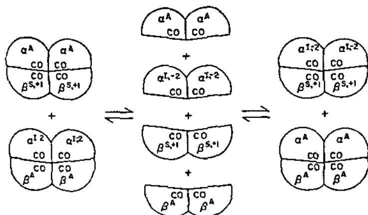


FIG. 6. Asymmetrical dissociation and recombination of HbCO S and HbCO I. Recombination of the subunits (centre) that result from asymmetrical dissociation to form HbCO A and a doubly abnormal molecule (right) in addition to the original components (left) accounts for the result shown in Fig. 5.

exchange of subunits between HbCO S and HbCO I, and asymmetrical recombination of these species accounts for the observed changes (Fig. 6). The electrophoretic mobility of HbCO I indicates an abnormality in net charge of close to -4. Chemical analyses have shown that haemoglobin I is abnormal in the α chain (Murayama and Ingram, 1959; Ingram, 1959); therefore, HbCO I can be designated $[\alpha_2^I\beta_2^A]^{-4}$. Asymmetrical dissociation of HbCO S and HbCO I would result in a mixture of α_2^A, $\beta_2^{S,+2}$, $\alpha_2^{I,-4}$, and β_2^A. The products of recombination would be $\alpha_2^A\beta_2^A$, $[\alpha_2^I\beta_2^S]^{-2}$, and the original components. $\alpha_2^A\beta_2^A$ is HbCO A, and $[\alpha_2^I S_2^S]^{-2}$ is a molecule with a net charge between

those of HbCO A and HbCO I. Recombination of HbCO C with a mixture of HbCO A and I yielded an increase in the component with the mobility of HbCO A without any new electrophoretic component. The observed increase in the electrophoretic component corresponding to HbCO A is consistent with asymmetrical recombination of HbCO C with HbCO I to form $\alpha_2^A\beta_2^A$ and $\alpha_2^I\beta_2^C$, which have the same net charge.

These experiments confirm the location of the defect of haemoglobin I in the α chain and suggest a simple method for identifying the altered chain of any electrophoretically abnormal haemoglobin. A haemoglobin in which the altered chain is known, for example haemoglobin S, is recombined with another abnormal haemoglobin. If the other haemoglobin is defective in the same chain as haemoglobin S, no change in composition will be observed. If the haemoglobin is defective in the other chain, a change in composition will occur. Samples from the first known families with haemoglobin D (Itano, 1951), haemoglobin I (Rucknagel, Page and Jensen, 1955), haemoglobin J (Thorup *et al.*, 1956) have been tested by this criterion. The results indicate that these samples of haemoglobins D and J carry their defects in the β chain and that the haemoglobin I reported by Rucknagel, Page and Jensen is, like the haemoglobin I of I. R. Schwartz and associates (1957), defective in the α chain (Itano and Robinson, unpublished studies).

Discussion

The two pairs of polypeptide chains and the four haems of a molecule of human adult haemoglobin are arranged symmetrically about a dyad axis (Perutz, Liquori and Eirich, 1951). Dilute acid dissociates a molecule, $\alpha_2\beta_2$, into subunits α_2 and β_2, each of which contains two haems. The symmetry relationships and the composition of the subunits signify that one haem is associated with each polypeptide chain (Singer and Itano, 1959). If, as suggested by the sedimentation data of Field and O'Brien (1955), the subunits are the same size, the α chain and the β chain are structural analogues of myo-

globin with one haem directly bound to a folded polypeptide chain of 16,000 molecular weight.

The observed mode of splitting indicates that in dilute acid the pairing of identical chains is much stronger than the association of α_2 subunits with β_2 subunits. We have not, however, observed association of analogous pairs to form species such as α_4^A, β_4^S, or $\beta_2^S\beta_2^C$ in our recombination experiments with haemoglobins A, S, C and I. We conclude that complementarity between α_2 and β_2 subunits is considerably greater than that between identical or analogous subunits and that species composed of four α chains or four β chains do not form to any appreciable extent if equivalent concentrations of α_2 and β_2 subunits are present. On the other hand, recombination of a mixture of HbCO A and canine HbCO produced a mixture of the original components and two new components (Itano and Robinson, unpublished studies). The apparent affinity between chains of the haemoglobins of different species suggests a physical definition based on complementarity for the α and β chains of haemoglobins in which the N-terminal sequences are unknown or differ from those of haemoglobin A.

One of the initial objectives of these studies was to determine whether or not the absence of hybrid molecules in heterozygous cells is due to physical incompatibility of unlike half-molecules. The structural requirements of physical incompatibility have been discussed in detail (Itano and Singer, 1958). Our recombination experiments do not test the incompatibility postulate since dissociation in dilute acid does not yield single chains or half-molecules of the type $\alpha\beta$. Synthesis of chains in stable, identical pairs, remains a possible explanation for the absence of hybrid chain pairs or molecules. Identical chains may be synthesized simultaneously or in rapid succession on the same template and fold into a relatively undissociable paired configuration before contact occurs with chains synthesized on other templates.

Recombination of HbCO I with HbCO S or HbCO C resulted in apparent formation of HbCO A and a haemoglobin

composed of two different pairs of abnormal chains. The formation of a normal molecule from the normal portions of two inherited abnormal molecules suggests an analogy with interallelic complementation in heterokaryons of *Neurospora crassa* (Giles, Partridge and Nelson, 1957; Fincham and Pate-

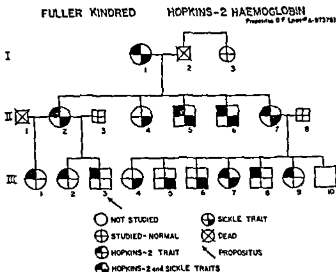


FIG. 7. Pedigree of family showing independent segregation of genes controlling the synthesis of haemoglobin S and Hopkins-2 haemoglobin. (From Smith and Torbert, 1958 Reproduced by permission of the original authors and the Editors, *Bull. Johns Hopk. Hosp.*) Individual III–10, previously not studied, has been found to have both Hopkins-2 and sickle traits (Smith, personal communication)

man, 1957; Catcheside and Overton, 1958). These results lead naturally to the question of whether or not the α and β chains are controlled at the same or different genetic loci. Whatever the nature of genetical control, the α_2 and β_2 subunits would combine at random if they are released independently from the site of synthesis. Independent synthesis of abnormal α_2 and β_2 subunits and their normal analogues in the same cell would result in four molecular species. These may appear as three or four electrophoretic components,

depending upon the relative net charges of the abnormal subunits.

Smith and Torbert (1958) have studied a family in which haemoglobin S and an abnormal haemoglobin designated Hopkins-2 occur. The pedigree indicates independent segregation of the genes controlling the two abnormal haemoglobins (Fig. 7). Apparent double heterozygotes for haemoglobins S and Hopkins-2 have three haemoglobin components by filter paper electrophoresis. We have recombined a mixture of HbCO A and HbCO Hopkins-2 with HbCO C, HbCO J, and HbCO S and have obtained results which suggest that Hopkins-2 haemoglobin is normal in the β chain. Our experience with haemoglobins C and I suggests the possibility that the component migrating as haemoglobin A in the analyses of Smith and Torbert may include a molecular species composed of the abnormal pair of β chains of haemoglobin S and an abnormal pair of α chains of Hopkins-2 haemoglobin as well as haemoglobin A. H. C. Schwartz and co-workers (1957) have described a family in which the pedigree indicates non-allelic control of haemoglobins S and G. Absence of haemoglobin A in double heterozygotes for the genes controlling haemoglobins S and G in this family suggests that the mechanism for control of synthesis of haemoglobin G is not analogous to that of Hopkins-2 haemoglobin.

Summary

Human adult haemoglobin, $\alpha_2\beta_2$, dissociates asymmetrically in acid into two unlike subunits, α_2 and β_2, each of which consists of an identical pair of polypeptide chains and two haems. Exchange of subunits occurs when an acid-dissociated mixture of two inherited forms of adult haemoglobin is recombined by neutralization. The new species formed by acid recombination of a mixture such as $\alpha_2^{A^*}\beta_2^{A^*}$ and $[\alpha_2^{A}\beta_2^{C}]^{+4}$ are $\alpha_2^{A}\beta_2^{A^*}$ and $[\alpha_2^{A^*}\beta_2^{C}]^{+4}$, not $[\alpha^{A^*}\alpha^{A}\beta^{A^*}\beta^{C}]^{+2}$; therefore, the process is analogous to complementation of gene-products. Normal adult haemoglobin and a haemoglobin composed of

two different pairs of abnormal chains are apparently produced by asymmetrical recombination of a haemoglobin defective in the α chain and a haemoglobin defective in the β chain. The altered chain of an abnormal haemoglobin can thus be identified by recombining the haemoglobin with another abnormal form in which the altered chain is known. The possible significance of these *in vitro* findings to problems of genetical control of haemoglobin synthesis has been discussed.

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DISCUSSION

Burnet: Have you any information about the serological differences amongst the various haemoglobins?

Itano: Foetal haemoglobin is easily distinguished serologically from adult haemoglobin; however, haemoglobins A and S are difficult to distinguish. Goodman and Campbell using both rabbit and chicken antisera have reported evidence for minor differences in antigenicity between haemoglobins A and S [Goodman, M., and Campbell, D. H. (1953). *Blood*, **8**, 422].

Lederberg: Have you looked for iso-antibodies after transfusion in Man?

Itano: No.

Lederberg: Would that not be the most sensitive method of looking for a difference between the haemoglobins?

Ceppellini: But there is absolutely no evidence of iso-antibodies in Man; they are only in rabbits

Lederberg: That is what you would expect. The two proteins are almost identical and quite different from the rabbit's.

Ceppellini · But S and A are too similar to be antigenic. I agree that isoimmunization is always more sensitive.

Rossi-Fanelli: I wonder if, following acid dissociation, studies have been made of the physiological properties of the reconstituted haemoglobin, for example, the combination with oxygen or other substrates, because acid dissociation may alter the molecule. Secondly, have you tried dissociating with urea?

Itano: Treatment with acid at low pH does alter the molecule, and the alteration is accompanied by a marked change in absorption spectrum to that of acid-denatured haemoglobin. Dissociation into two subunits at pH 4–6 appears to be reversible according to physical criteria, and the reconstituted molecule has the electrophoretic mobility and absorption spectrum of native haemoglobin We have not examined the physiological properties of reversibly dissociated haemoglobin, nor have we attempted recombination experiments with urea-dissociated haemoglobin. It has been my experience in the past that removal of urea from a solution of urea-dissociated

haemoglobin A results in precipitation of a large fraction of the haemoglobin; therefore dissociation with urea is probably not as readily reversible as dissociation with dilute acid.

Brenner: Have you done any recombination experiments with haemoglobin M?

Itano: I have not worked with haemoglobin M although the properties of one of the two chemically different forms of haemoglobin M suggest the application of recombination experiments. About half of the haem groups in this haemoglobin have abnormal chemical reactivities [Gerald, P. S., and George, P. (1959). *Science*, 129, 393]. It would be possible to determine whether these abnormal haem positions are associated with a particular pair of chains by recombining haemoglobin M with abnormal haemoglobins in which the defective chain has been identified.

Lederberg: Dr. Itano, you referred to the Hopkins-2 family as evidence of interchange of chains *in vivo*. Could you clarify the argument?

Itano: The familial evidence published by Smith and Torbert shows that haemoglobins S and Hopkins-2 are controlled by genes that segregate independently. According to their electrophoretic analyses individuals with both abnormal genes have three haemoglobin components, the two abnormal forms and haemoglobin A. On the other hand, individuals with haemoglobins S and C, which are chemically defective in the same chain and which according to familial studies are under the control of allelic genes, do not have any haemoglobin A. Our recombination experiments suggest that Hopkins-2 is abnormal in the α chain. The findings of Smith and Torbert can be reconciled with the structural composition of the haemoglobin molecule if we postulate that the component that migrates like haemoglobin A in apparent double heterozygotes includes, in addition to haemoglobin A, a double abnormal molecule composed of the abnormal β chain of haemoglobin S and the abnormal α chain of haemoglobin Hopkins-2, the charge defects of the abnormal chains cancelling out to produce a new species with the same net charge as haemoglobin A. Since we are looking at haemoglobin after completion of synthesis, we cannot state whether the apparent formation of four molecular species occurs by synthesis of two different pairs of α chains and two different pairs of β chains followed by random association of these pairs or by synthesis of haemoglobin S and Hopkins-2 followed by intracellular interchange of chains. The familial evidence shows that the genes for S and Hopkins-2 segregate independently; therefore it would appear more likely that synthesis of the different chains on independent templates precedes random association.

Lederberg: The very fact that there are more than two components is the basis of your argument?

Itano: The presence of more than two components and the mobility of the third component, which suggests that it may be a mixture of haemoglobin A and a doubly abnormal molecule, are the chemical bases of my argument. It will be necessary to isolate the third component and examine it chemically for the presence of both abnormal chains in order to test my hypothesis.

THE GENETICAL CONTROL OF PROTEIN STRUCTURE: THE ABNORMAL HUMAN HAEMOGLOBINS*

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IT is widely supposed nowadays that the structure of a normal protein is completely determined by the structure of a Mendelian gene. A gene passes on unchanged from generation to generation and, as a result of the action of such a gene, in each generation normal protein molecules of identical structure are produced. Just how such proteins are made we do not as yet know and this is a subject for further research. We do know, however, that, when a mutation occurs and when as a result a particular gene is changed, then an abnormal protein is produced. We also know that such abnormalities can be passed on from generation to generation through the agency of a gene. The direct study of the chemical relationship between the gene and the protein which that gene controls is as yet much too difficult a task. It is, however, possible to study chemically some inherited abnormalities of proteins. In particular in the case of the various abnormal human haemoglobins (Itano, 1956, 1957; Lehmann, 1957*a* and *b*) we have one of the clearest instances of inherited abnormalities in a protein which can produce, by virtue of their abnormality,

* A considerable amount of the material published here has already appeared in *Brit. med Bull.* (Ingram, 1959*a*).

serious diseases. Some, though not all, of the abnormal human haemoglobins are the causes of haemolytic anaemias. By studying the chemical changes which distinguish these molecules from those of normal haemoglobin, it is possible to learn something about the chemical effect which mutation of a Mendelian gene produces and at the same time to investigate what it is in the protein which causes the disease. When considering the kind of alterations which a protein molecule can undergo as a result of mutation, three possibilities come to mind: are the differences due to changes in the amino-acid sequence of the polypeptide chain in the protein, or are they due only to changes in the folding of such a polypeptide chain revealing or masking some groups; or are both factors operating? It has been shown that there are certainly changes in the amino-acid sequence (Ingram, 1956, 1957; Hunt and Ingram, 1958*b*). The second or third alternatives have not yet been ruled out.

Whilst the abnormal human haemoglobins form at present the most convenient system for studying these questions, a good many other examples of inherited protein "abnormalities" are known For example, the haemoglobins of cattle, sheep and goats are each known to occur in two different forms (see Itano, 1957). There is no indication in these animals that either form is connected with a disease though they can differ in their affinity for oxygen; similarly the two β-lactoglobulins of cattle (Aschaffenburg and Drewry, 1957). Various forms of the enzyme tyrosinase in the mould *Neurospora* have been found (Horowitz and Fling, 1956) and a similar situation exists concerning the enzyme tryptophan synthetase (Yanofsky, 1956). The human haemoglobins are preferred for this work, because many mutant forms are known, because it is relatively easy to prepare the pure protein and, last but not least, because the molecular weight of the protein is not too large.

The best known of these abnormalities of human haemoglobin is the one associated with sickle-cell anaemia. It has been known for a long time that the disease occurs in two rather

distinct forms, a mild anaemia known as sickle-cell trait, and the severe sickle-cell anaemia itself. It became apparent that this is an inherited disease, though the relationship between the two forms and their inheritance was for a long time obscure. In 1949 two most important advances were made. Neel (1949) showed that the disease is inherited in the manner of a single Mendelian gene, that the mild disease was shown by people who are heterozygous for this form of the haemoglobin gene, and that the severe anaemia showed itself in homozygotes who carried two abnormal haemoglobin genes. The second advance was that made by Pauling and co-workers (Pauling, Itano, Singer and Wells 1949; Pauling, 1954) who showed that the haemoglobin of sickle-cell anaemia differs electrophoretically from the haemoglobin of normal subjects, indicating a chemical difference between the two types of molecule. They also showed that people who carry both the normal and abnormal human gene produce both normal and sickle-cell haemoglobin, a state corresponding to the sickle-cell trait. Conversely the sickle-cell homozygotes produce no adult haemoglobin. These findings showed the direct correspondence between mutations in a gene and chemical changes in the protein which that gene controls. It is known (Perutz, Liquori and Eirich, 1951) that the haemoglobin molecule is composed of two identical half-molecules. Since many chemical studies (see Ingram, 1956, 1957) such as amino-acid analyses failed to show any significant difference between these two haemoglobins although physical differences could be observed, the alteration is likely to be a small one.

Since 1951 many other abnormal human haemoglobins have been discovered (Itano, 1956, 1957; Lehmann, 1957*a* and *b*). The most commonly occurring haemoglobins, after haemoglobin S of sickle-cell disease, are haemoglobin C and haemoglobin E, both of which reach quite high frequencies in some countries such as Ghana and South East Asia, respectively. All the other abnormal forms are rare except in some localized communities. Most people are expected to have identical and normal haemoglobin molecules but, in a few, minor variants

might result from mutation. Most of these, arising as mutants, would be expected to occur only rarely; a few, however, that are favoured by natural selection (see Itano, 1957), would be expected to reach quite high frequencies. In this last class are the haemoglobins S, C and E.

The work of Perutz and his collaborators (see Kendrew and Perutz, 1957) has shown that the molecule of horse haemoglobin is an ellipsoid of the following dimensions: 55 × 55 × 70 Å. Human haemoglobin is thought to have a rather similar shape, with a molecular weight of 66,700. X-ray crystallographic studies have shown that the molecule is composed of two equal parts; therefore, if we can localize a chemical alteration in one half of the molecule, it is likely to be duplicated exactly in the other half. In each half-molecule there are two different peptide chains which are called the α chain and the β chain (Rhinesmith, Schroeder and Martin, 1958). In the whole molecule there are therefore two α chains and two β chains coiled up in some definite, but as yet unknown, manner. Normal adult haemoglobin—haemoglobin A—may therefore be written as $\alpha_2^A\beta_2^A$.

In the half-molecule there are approximately 300 amino acids amongst which we must search for the chemical difference between a normal and an abnormal haemoglobin molecule. Since this difference may involve as little as one charged group and therefore only one out of 300 amino acids, it is necessary to break down the molecule into small peptides, and to examine the mixture of peptides which results.

The initial degradation of the haemoglobin molecule is brought about by the action of the proteolytic enzyme, trypsin (Ingram, 1958). Before the digestion it is necessary to denature the protein, here accomplished through the action of heat. Trypsin rapidly digests the heat-treated haemoglobin, splitting the peptide chains at the points where the amino acids lysine and arginine occur, and nowhere else. Since there are approximately 26 molecules of lysine and arginine in each half-molecule of haemoglobin, the resultant mixture of peptides contains approximately 28 fragments. The mixture of

peptides obtained from the digestion of normal haemoglobin has now to be compared with a mixture from sickle-cell haemoglobin.

Separation of these peptides was achieved by a combination of filter-paper electrophoresis and chromatography on the same piece of paper (Ingram, 1958). Such maps, or finger-prints as they are now called, have been prepared from normal haemoglobin and sickle-cell haemoglobin (Fig. 1). All the peptides occupy the same position and therefore probably have the same chemical structure in the two cases, with the exception of a single peptide. This, the number 4 peptide, occupies a new and different position in the sickle-cell haemoglobin finger-print, indicating that it carries a difference in chemical constitution. The new position of this peptide is in part due to an alteration in the number of electric charges

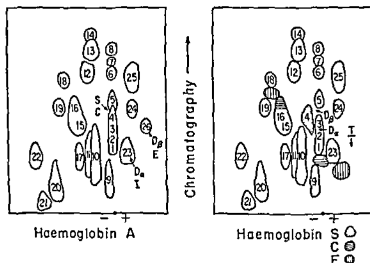


FIG. 1. Summary tracing derived from "finger-prints" of normal and abnormal human haemoglobins. The peptides of normal haemoglobin which will carry the mutational changes so far recorded are indicated and so are the new positions which they occupy in the abnormal proteins Peptide 23 changes in haemoglobin I.

which this peptide carries. This finding agrees well with the known electrophoretic behaviour of the parent protein.

A considerable portion of the protein molecule is not digested by trypsin. This "core" had to be digested with another enzyme, chymotrypsin, to yield a mixture of peptides characteristic of that core (Hunt and Ingram, 1958*a*). Examination of these peptides by finger-printing showed the cores of normal and sickle-cell haemoglobin to be similar; so did the examination by qualitative amino acid analysis of all the peptides of the original trypsin finger-prints. It seemed

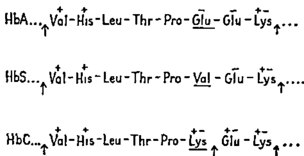


FIG. 2. Amino acid sequences of the peptides 4 in haemoglobins A, S and C.

that the only chemical difference between the two kinds of haemoglobin molecule resided in the two different number 4 peptides. Therefore, the next step was the determination of the chemical structure of these two peptides.

These number 4 peptides were examined by the methods of partial acid hydrolysis and end-group determination of the fragments, using already well-known procedures. As a result both the normal and the sickle-cell number 4 peptide were found to contain a chain of eight amino acids (Fig. 2). These were identical in the two cases but for the sixth amino acid. A glutamic acid residue which occurred in the normal peptide had been replaced by valine; that is to say, a negatively charged group had been replaced by a neutral group, thus

explaining the difference in electric charge and the difference in electrophoretic mobility of the parent protein. This substitution of only one of 300 amino acids appears so far to be the only structural alteration caused by the mutation of this haemoglobin gene. Whether or not the change induces also an alteration in the folding of the polypeptide chains has not yet been determined. Since this peptide begins with the sequence valyl-histidyl-leucyl (Hunt and Ingram, 1959) it is likely to be the N-terminal peptide of the β-chain of haemoglobin (Rhinesmith, Schroeder and Martin, 1958).

These findings do not in themselves explain the abnormally low solubility of the reduced sickle-cell haemoglobin (Perutz and Mitchison, 1950) However, it is likely that the solubility of a protein depends upon the distribution of positive and negative charges on its surface, and to disturb this pattern, as has happened in sickle-cell haemoglobin through the removal of glutamic acid, is quite likely to result in a change of solubility. We may deduce that the glutamic acid which is affected by this mutation occupies a key position in the three-dimensional structure of the protein.

Haemoglobin C is of interest because it occurs with high frequency and also because there is some genetical evidence (see Itano, 1957; Neel, 1956) that the two mutations—haemoglobins S and C—occur in the same locus of the haemoglobin gene or at closely linked loci.

Samples of pure haemoglobin C were subjected to the same method of trypsin and chymotrypsin digestion, followed by finger-printing of the peptide mixtures obtained. It was most interesting to discover in finger-prints of haemoglobin C that the same number 4 peptide which had changed in sickle-cell haemoglobin had changed also in haemoglobin C (Hunt and Ingram, 1958*b*). It gave rise this time not to one new peptide but to two new ones; once again the same glutamic acid residue or normal haemoglobin had been replaced, but this time by the amino acid lysine. This new lysine offered a new point of attack to trypsin, causing the number 4 peptide to appear as two fragments. A change from glutamic acid to

lysine involves a difference of two units of electric charge, twice as much as in the case of sickle-cell haemoglobin (Fig. 2). This is in good agreement with the fact that haemoglobin C shows an electrophoretic difference which is roughly twice as great as that of sickle-cell haemoglobin.

The amino-acid substitutions of these two abnormal haemoglobins which appear to be the only chemical changes detectable in these molecules illustrate very well the fine action of gene mutations. These findings also reinforce the genetical evidence that the two mutations occur in similar places on the gene, since they affect the same amino acid. It is clear how profoundly the physical and physiological properties of a protein molecule are affected by such simple amino-acid substitutions, since haemoglobin C does not sickle.

Similar investigations (Hunt and Ingram, 1959) are under way on yet another abnormal haemoglobin, haemoglobin E (Itano, 1957; Lehmann, 1957*a* and *b*), which occurs frequently in Burma. Here again it is found that one of the peptides of a trypsin digest has undergone a profound change and that a glutamic acid of normal haemoglobin has been replaced by the amino acid lysine, involving a change of two units of electric charge. However, it is very interesting to note that, in this mutation, the glutamic acid is carried in peptide number 26 and not in peptide number 4. Although the type of substitution is similar to that found in haemoglobin C, yet a different part of the molecule is involved. It is likely that this represents the only change in the molecule.

Several other abnormal haemoglobins are currently being investigated by these methods in order, on the one hand, to illustrate the range of chemical alterations caused by mutations, and on the other hand to find the chemical basis for these disorders. In particular haemoglobin D (Itano, 1957), one of the rare haemoglobins, has shown that it too carries peptide changes. Although different samples of haemoglobin D have similar electrophoretic behaviour, yet it appeared in two samples (called D_α and D_β) from different individuals that two *different* peptides show chemical changes (Benzer, Ingram

and Lehmann, 1958). This shows clearly, as had been suspected before, that characterization of the abnormal haemoglobins by electrophoresis alone is not sufficient. Fig. 3 illustrates diagrammatically the peptide changes which we believe characterize these abnormal haemoglobins, including also haemoglobin I (Murayama and Ingram, 1959), another rare haemoglobin.

It was mentioned earlier that there are two different

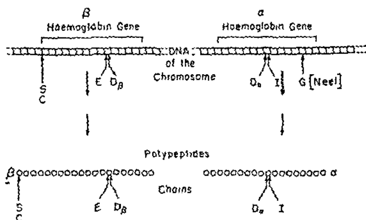


FIG. 3. Schematic representation of the hypothesis which relates the changes in a protein with mutations of the gene.

polypeptide chains in the haemoglobin molecule, an α chain and a β chain. It is of interest to determine which of these two chains carries the various amino-acid changes described above. The presence of two such chains tempts one to postulate that there is not one haemoglobin gene but two (Fig. 3), each responsible for making one of the haemoglobin polypeptide chains. That this might be so has been postulated earlier by Neel and his colleagues (Schwartz *et al.*, 1957) on the basis of his study of a family in which both sickle-cell haemoglobin and haemoglobin G (Neel) appeared. A similar suggestion has been made by Smith and Torbert (1958) for a pedigree involving the haemoglobin "Hopkins-2".

It was possible to separate the haemoglobin chains by electrophoresis in the presence of a detergent or by urea chromatography on an ion-exchange resin (Ingram, 1959*b*). Finger-printing showed that roughly half the original peptides were present in each.

Since it is known which haemoglobin peptides are defective in the various abnormal haemoglobins mentioned in this article, we can assign them to one or other polypeptide chain. Haemoglobins D_β and E change in the same peptide, number 26, though not necessarily in the same amino acid. Haemoglobins S (sickle cell) and C change in the same amino acid of peptide 4 at the beginning of the β chain. It is found that the haemoglobin S and C mutations and the haemoglobin D_β and E mutations occur on one identified as the β chain. This is in agreement with the postulate of Aksoy and Lehmann (1957) that the S and E mutations are linked By contrast, the other chain carries the mutational alteration characteristic of haemoglobins D_α and I, which again are contained in the same peptide fragment—peptide number 23.

In this connexion it is of particular interest that Vinograd, Schroeder and Hutchinson (1959) have shown by an ingenious "hydridization" experiment involving haemoglobin labelled with ^{14}C that the α chains of haemoglobins A and S are interchangeable and therefore similar. On the other hand, the β chains are not interchangeable and must carry the mutational alteration which characterizes haemoglobin S. These two haemoglobins may be written as—$\alpha_2{}^A\beta_2{}^A$(HbA) and $\alpha_2{}^A\beta_2{}^S$ (HbS). It is very satisfying that two such different methods as this one and the one described in the previous paragraph have given the same result.

It is also interesting to note that Jones and co-workers (1959) have found that haemoglobin H is composed of 4 β chains so that its structure is provisionally written as $\beta_4{}^A$, indicating that its β chains are interchangeable with those of haemoglobin A. This is a most interesting finding since the inheritance of haemoglobin H has always been puzzling. It is associated with thalassaemia and is only expressed when the

person is apparently homozygous for H, together with the production of haemoglobin A. Since H is now β_4^A, it seems that the condition is an interference with the production of α^A chains leading to an excess of β^A chains which associate to form β_4^A. One wonders whether the α chains of the haemoglobin A found in persons who also show haemoglobin H are really normal in their detailed chemical structure. The rather unlikely possibility also exists that the β chains which make up the haemoglobin H molecule and which resemble normal β chains in their gross structure do differ in some detail important enough to lead to the overproduction of this type of chain.

It is now important to look for the other phenomenon—the occurrence of a haemoglobin which can be written as α_4^A. Perhaps the "Lepore" haemoglobin described by Gerald and Diamond (1958) might be such a molecule, since its mode of inheritance is reminiscent of haemoglobin H and since it has the necessary slower electrophoretic mobility. It is also conceivable that some of the so-called minor components—though not Kunkel's A_2—of haemoglobin are hybridization products of the type $\alpha_3^A\beta^A$, $\alpha^A\beta_3^A$, etc.

Another recent development is the finding by one of us (Hunt, 1959) and by Vinograd and Schroeder (1959) that human foetal haemoglobin has two α peptide chains which resemble those of adult haemoglobin A. The other two chains are different and are called the γ chains. This is a most important finding, since it infers that one of the postulated two haemoglobin genes—the "α" gene—remains active during the changeover from foetal to adult haemoglobin production and that the "choice" is made between activating the "γ-chain" gene and the "β-chain" gene. It is also possible that a duplicate set of "α genes" exists, which may or may not be identical in fine detail with the "α gene" concerned in adult haemoglobin manufacture. It will, therefore, be interesting to see whether the α^F chain is like the α^A chain in detail, but this seems likely. It has always been somewhat puzzling in people homozygous, for example, for haemoglobin S who often also

make haemoglobin F, that the haemoglobin F is apparently quite normal. This is now readily explicable, since the β chain which is abnormal in haemoglobins S and C simply does not occur in haemoglobin F. Hence this protein is normal. If, on the other hand, it is true that the same "α-chain" gene is used for making the α chains that go into both haemoglobin F and the adult haemoglobins, then we would expect to find in a newborn destined to produce haemoglobin I that its haemoglobin F is abnormal. This would be because it is the α chain that is altered in haemoglobin I. Such a situation, which is probably rare enough, would provide a good test of the idea that the same α chain gene is used for making the foetal and the adult haemoglobin. Since it is possible that these are made in the same cells, we may have here a possible approach to the important problem of how genes are turned "on or off" during cell differentiation.

These findings enable us to build up a tentative picture of the way in which the structure of both normal and abnormal haemoglobin molecules is determined (Fig. 3). Two genes appear to be involved which may or may not be on the same chromosome. The chemical structure of each of these genes is thought to determine the amino-acid sequence of the corresponding polypeptide chain. On the basis of this picture any chemical alteration of the gene—a mutation—would result in a chemical change of the corresponding polypeptide chain and therefore of the protein molecule. The fact that definite amino-acid substitutions in one or other haemoglobin chain have been found as the result of such gene mutations supports the hypothesis strongly.

Many mutations are theoretically possible with each haemoglobin; each one would induce a chemical change in the protein molecule, in this case haemoglobin. Such chemical changes may or may not be manifested by physiological effects. Some which occur in less important parts of the molecule, and particularly those involving uncharged groups, might never be noticed clinically. Many others might well turn out to be lethal and would not be observable for that reason. In looking

at the abnormal human haemoglobins we must realize that we are looking at a highly selected group of mutations; selected, that is to say, both for their clinical effects and for their electrophoretic behaviour. They do not, therefore, form a truly representative sample to illustrate the chemical effect of gene mutations; nevertheless, their study has given valuable insight into the action of a gene and in particular into the fine control exercised by a gene.

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DISCUSSION

Pontecorvo: With regard to terminology, could we use a completely novel term for the case in which two mutant proteins have one or more amino acids changed in the same position along the sequence: "isotopically mutant"?

Brenner: We have already proposed one and have called them "isocodonic": the "codon" is the unit of genetic information that determines a single amino acid. This is not meant seriously!

Neel: This GS-thalassaemia family which has been mentioned already could be discussed at some length, because we may have here a new phenomenon which is quite exciting. In this family (Schwartz *et al.*, 1957, *loc. cit.*) an individual with the sickle-cell trait married a woman who appears to be heterozygous for the genes responsible for haemoglobin G and thalassaemia. There resulted a son with an anaemia clinically identical with sickle-cell anaemia. By electrophoresis he has either haemoglobin G—or a G-like haemoglobin—and haemoglobin S. He married a normal woman Their only child clearly has thalassaemia. Now on the basis of the absence of both G and S in this child, we concluded on the basis of a series of one that the most reasonable interpretation here was of non-allelism of these two genes. With the finding that the mutation responsible for haemoglobin G involves an amino acid substitution next to the amino acid position affected in haemoglobins S and C, a re-examination of this pedigree is obviously indicated. One possibility which emerges is of course that what was interpreted as G is in fact not G but possibly *a "new" haemoglobin which has been formed in the individual* heterozygous for the S gene and thalassaemia. If this is the case, then this new haemoglobin could be termed a "hybrid substance", such as Irwin drew attention to many years ago in doves

(Irwin, M. P. (1951). *In* Genetics and Immunology, ed. Dunn, L. C. New York: Macmillan). It is an interesting fact that in the woman mentioned earlier whom we thought to be heterozygous for the G and thalassaemia genes, there was an anomalous component which behaved very much like C or S, and in fact we drew attention to the possibility of a hybrid substance in this woman. If these are hybrid substances, then in the light of what we have heard about recombination in α and β chains we may now have an explanation of a phenomenon which has been commented on many times in the past.

Ingram: One possible way of explaining the inheritance of these abnormal haemoglobins in this particular family might be to assume that this case of thalassaemia involves mutation on the gene controlling the β chain, i.e. the same chain that is affected in haemoglobin S and in haemoglobin G; and that all the α chains (and genes) are completely normal. It may be that this is not the only explanation.*

Then the explanation would run as follows: here is a mother who makes nothing but haemoglobin G. We might suppose she has the β^G gene, she also has thalassaemia, and because of that she makes no haemoglobin A. Remember the alphas are all normal. She has a sister who has the same phenotype. She also has thalassaemia and haemoglobin G, and because of the thalassaemia she makes nothing but haemoglobin G. (This is the one that Hill and Schwartz of Salt Lake City have analysed.) The sisters are married to normal people. Let us take this critical situation here and consider only the β locus. This woman—postulated of genotype β^G/β^T—is married to a man who has sickle-cell trait, so he is β^A/β^S. The offspring of this marriage, the propositus, we believe to be β^S/β^T, with no haemoglobin G contrary to the findings originally published; remember that haemoglobins S and G are very close electrophoretically and are difficult to tell apart. The rest of this pedigree is perfectly straightforward, because the child of the propositus and a normal mother has a β^T—a thalassaemia—gene from his father and a β^A gene from his mother. The published finding which stands against the idea of the critical propositus having only β^S and β^T genes and making nothing but sickle-cell haemoglobin, is the reported solubility of his sickle-cell haemoglobin. The solubility of pure sickle-cell haemoglobin ought

* However, in that case we would postulate 4 different β chain genotypes in this family, making respectively β^A chains (normal), β^S chains (sickle cell), β^G chains (haemoglobin G) and β^T (hypothetical thalassaemic β chains). We would further postulate that the β^T chain is so drastically altered that it cannot be made. Such an alteration may or may not be electrophoretic; it would not be seen.

to be very low: in fact his is intermediate, more like a mixture of A and S, or S with some other haemoglobin, like G. It is conceivable that in this case a second component is present due to an excess of α chains being made. This person would then have both haemoglobin S and a new and unknown component—α_4^A, which electrophoretically would be almost indistinguishable from haemoglobin S, but would have quite a different solubility and explain the fact that the solubility of haemoglobin in this case is not low as it ought to be. When one looks at the published pictures of paper and boundary electrophoresis, this hypothesis is not unreasonable; there are certain bumps and shoulders in the diagrams. However, the brother of the propositus has *no* thalassaemia, his genotype would be β^s/β^o and again the electrophoretic and solubility data, etc., are consistent with the view that he does indeed have both haemoglobin G and S, and no α_4^A, no thalassaemia. The reason that the various haemoglobins in this family have been so difficult to distinguish chemically is due to the unfortunate circumstance that both haemoglobins G and S and the as yet hypothetical α_4^A have very similar electrophoretic mobilities.

Kalmus: Dr. Ingram might perhaps find time to look at some of the mouse literature. I think anaemias have been described in laboratory mice affecting the foetal, intermediate and adult generations of red cells both morphologically and in respect of their haemoglobin content. I should also like to know whether Dr. Ingram has any particular reason for using only trypsin to study variation in the α chains, and not other enzymes?

Ingram: There are other enzymes which can be used in this work, but they are not as specific as trypsin, and that means that you are likely to get apparent variations due to the presence of the products of incomplete digestion. Trypsin is still the best.

Lederberg. With regard to the chemical methods of determining allelism, in principle the unique products of mutant genotypes might have been specified already in the wild type. Does the mutation convey new information or switch the actual production to another locus already there? This seems to hold for the alternative production of haemoglobins A and F. One relevant datum would be the possible occurrence of trace amounts of mutant haemoglobins in "normal" bloods. I do not mean at all that this argument should be taken very seriously but it has to be considered in a rigorous analysis. What is the maximum level of alternative haemoglobins that could be present in the normal individual and yet not be detected by existing methods—is it as high as 1 per cent?

Ingram: I would guess that it is of the order of between one-tenth and one per cent.

Lederberg: Do you think it would be worth while to make a systematic search for residual amounts of variant haemoglobin?

Ingram: This is being done at the California Institute of Technology. There are a number of minor components of haemoglobin A apart from the two I have already mentioned which do occur and which are being investigated. There are, however, other ways in which these minor components could arise. The whole series of minor components is apparently repeated in haemoglobin S and haemoglobin C; in other words, it is possible that those are errors in the mechanism of production.

Lederberg: In the α chain, for example?

Ingram: Yes, they could be systematic errors. Or they could be different assortments of α and β chains. We have only heard so far of haemoglobins which are $\alpha_2^A\beta_2^A$ and β_4^A and α_4^A, but this is because nobody has looked for $\alpha_1^A\beta_3^A$ and $\alpha_3^A\beta_1^A$, etc.

Lederberg: Has β_4 been obtained by reassociation *in vitro*? Is β_4 an hypothetical or a real substance?

Ingram β_4^A is a real substance, it is haemoglobin H. Schroeder and Vinograd tell me that they have made β_4^S.

Brenner: No one has separated the half-molecules?

Ingram: Not yet.

Lederberg: You also have somatic mutation, of course, in the erythropoietic system, which might be expected.

Ingram: You would expect to find those in the series of minor components which change with the adult.

Lederberg: Then we can justify the idea that there are many genes controlling the series of haemoglobins. Now what criterion do you use to relate S to A as being the mutant of that particular β chain?

Ingram: This is purely through genetical studies.

Lederberg: You use genetical evidence after all to determine allelism!

Pontecorvo: There are certain replacements of amino acids which will give an electrophoretic difference and others which will not. What proportion of possible changes, if every amino acid could be changed, would be electrophoretically detected?

Brenner: If you were given a protein which could change at random, and if all amino acids occurred equally frequently in that protein, then 40 per cent of all possible changes would lead to charge differences. This would be subject to a scaling factor depending on the composition.

Ingram: Does this include changes in uncharged amino acids?

Brenner: Yes, all possible changes.

Smithies: It is of interest that in cattle three transferrins can be

detected in homozygotes [Smithies, O., and Hickman, C. G. (1958). *Genetics*, 43, 374], and when there is a change in an allele at the corresponding genetic locus apparently all three transferrins change [Ashton, G. C., and McDougall, E. I. (1958). *Nature* (*Lond.*), 182, 945], and so there is a mixture of 6 transferrins in heterozygotes.

Ingram: Do they differ perhaps only in molecular complexity?

Smithies: As far as I am able to tell from the limited amount of work we have done the three transferrins in the homozygotes differ with respect to charge but not with respect to size. Since there are three possible alleles at the cattle transferrin locus there are altogether 9 transferrins; any individual animal can have 3 transferrins (homozygotes) or 6 (heterozygotes).

Neel: One of the very interesting aspects of our present knowledge of the genetic control of the haemoglobin molecule involves the mixture of qualitative and quantitative gene effects which has been demonstrated at the *S–C* locus The qualitative effects so well reviewed here by Dr. Ingram, have recently overshadowed the picture. However, six years ago it was demonstrated by a number of investigators that individuals homozygous for either the haemoglobin *S* or *C* genes had a significantly decreased haematopoietic reserve [Crosby, W. H., and Akeroyd, J H. (1952). *Amer. J. Med.*, 13, 273; Terry, D. W., Motulsky, A. G., and Rath, C. E (1954). *New Engl. J. Med*, 251, 365; James, G. W, and Abbott, L. D. (1955). *Proc. Soc. exp. Biol.*, 88, 398]. It is a fact worthy of emphasis that what we assume to be a very small change in a gene—because it involves the substitution in a protein of a single amino acid—can also decrease so very markedly the amount of end product associated with that gene.

It is obviously a matter of considerable significance to biochemical genetics to explore the question of how often a point mutation combines both qualitative and quantitative effects in this manner. Some recent work on acatalassaemia, with which I have had a small association, is of interest in this respect. This trait, characterized by the absence of catalase from the erythrocytes, was first described by S. Takahara in 1952 (*Lancet*, 2, 1101). Thus far the condition has only been observed in Japanese, but the recorded segregating families, some 17 in number by now, are found throughout Japan, from which we may surmise that the gene is one of considerable antiquity in the Japanese Islands. The fact that 16 of the 17 segregating sibships so far described are the issue of consanguineous marriages, and the approximation to a 3 : 1 ratio of normal to affected in segregating sibships, leave little doubt that the condition is due to a rare recessive gene. Recently, Nishimura and co-workers (Nishimura, E. T., Hamilton, H. B., Takahara, S., Kobara, T. Y., Ogura, Y., and Doi,

K. (1959). *Science*, in press) have demonstrated that in the (heterozygous) parents and some siblings of affected individuals, catalase values are, within the limits of technical error, half normal, This is, I believe, the first biochemical carrier state in which the heterozygote falls so precisely intermediate between the two homozygotes. The detailed analysis of the biochemistry of this situation has scarcely begun and, moreover, because of the relatively small amounts of catalase in the blood, presents certain difficulties. Nevertheless, I am tempted to speculate that ultimately we will find in this case that the mutant gene results in the elaboration of an altered protein-lacking enzymic activity but, in contrast to the findings as regards haemoglobin, a protein formed at the same rate as the normal enzyme. This postulate seems the most logical means of accounting for the apparent lack of any dominance phenomenon, although other possibilities, such as biochemical inactivity on the part of the mutant gene, of course cannot be excluded.

Incidentally, although acatalassaemia is a rare disease, on the assumption of genetic equilibrium it can readily be calculated that in Japan, with a population of some 88,000,000, there should be several hundred thousand heterozygous carriers of the gene. Critical data on the implications of this carrier state for general health are lacking; there are no obvious ill effects. On the other hand, should a situation arise where normal catalase values were important—as in the inactivation of bacterially-produced peroxides when other defence mechanisms were for some reason depressed—carriers might be at a disadvantage, and a concealed biochemical difference between individuals become of real biological importance.

Itano: It is necessary to think in terms of production per cell or allele in considering efficiency of genetic control of protein synthesis Since vertebrate haemoglobin occurs entirely in red cells, it is a simple matter to combine haematological and chemical data to calculate the average production per cell or allele. On the other hand, a normal concentration of a serum enzyme or of haemoglobin per 100 ml. of blood may arise from a compensatory increase in the number of subnormally efficient cells. Thalassaemia minor, in which an abnormally high concentration of microcytic cells may result in a normal overall haemoglobin concentration, represents an example of such a compensatory mechanism.

Siniscalco: Dr. Ingram, you found two or three different finger-print patterns when studying haemoglobin D from different sources. Did you find any difference in haemoglobins C or S from different sources? Secondly, is the fine structure of haemoglobin A_2 from normals just the same as that of haemoglobin A_2 from thalassaemic individuals?

Ingram: We do not know the answer to the second question. These are preliminary experiments on haemoglobin A_2, and we are at present working only with normals; we shall be working with the thalassaemics in due course. All sickle-cell haemoglobin samples which I have had from different parts of the world have turned out to be the same. However, there are reports of different types of haemoglobin S, e.g. of a strange one in St Louis, and there are some aberrant haemoglobins C. Eventually we hope to examine these also.

Kalmus: It seems to me that the experiments on complex-formation between human and animal haemoglobins as reported by Dr. Itano should be extended to some nearer relatives of Man such as apes and anthropoids; complex formation might also be between human haemoglobin and the haemoglobin of very remotely related forms. I think I am correct in saying that Keilin in Cambridge some time ago described the occurrence of haemoglobin in fungi.

Ceppellini· The reports presented by Itano and Ingram show beautifully how the chemical analysis of the phenotype at the molecular level can help in the understanding of the genetic facts, chiefly when the evidence deriving from formal analysis, as in the case of Man, is so scanty and difficult to collect

Nonetheless, the final proof of a genetic hypothesis can only come from the study of pedigrees For instance, on the basis of the S–I family of E. W. Smith and J V. Torbert (1958, *Bull. Johns Hopk Hosp.*, **101**, 38), one could not avoid the conclusion that Hb A, notwithstanding its chemical homogeneity, had to be the product of at least two distinct functional genes, acting in parallel, i.e the molecule of Hb A had to be at least "digenic" (Ceppellini R. (1959) *Acta Genet. med. (Roma)*, suppl. 2) Now this conclusion fits very well with the differentiated structure of the Hb half-molecule into the two peptides, α and β, which seem to be the (relatively) immediate products of two different genes, certainly non-allelic and possibly not linked. One other gene, different from the ones which are identified through the α and β mutations, is probably responsible for the synthesis of the minor slow-moving fractions A_2 and B_2.

A_2, which has electrophoretic mobility the same as E, is present in all normal adults as a small percentage (around 2 5 per cent) of the total haemoglobin. It is also present, at least qualitatively unmodified, in S–, C– thalassaemia-homozygotes; thus it cannot be regarded as the specific product of any one of the genes involved in the synthesis of adult haemoglobins. Two hypotheses have been put forward [Kunkel, H. G., Ceppellini, R , Muller-Eberhard, U., and

Wolff, J. (1957.) *J. clin Invest.*, 36, 1615]: (1) A_2 is a non-specific by-product of the same metabolic pattern which produces the adult haemoglobins; (2) A_2 is the specific product of a metabolic pattern controlled by an entirely different locus. But a locus can be formally identified only through mutation, i.e. at least two segregating alternative phenotypes must be known. Now we have identified a new minor haemoglobin component, B_2, which seems to be the product of a rarer allele of the A_2 locus (Kunkel, H. G., Ceppellini, R., Dunn, L C., and Firsheim, L. (1959). Unpublished data).

Among American Negroes, the haemoglobin pherogram (starch block, pH 8 2) shows in a few subjects a small spot of pigment moving behind the C position, therefore much slower than A_2.

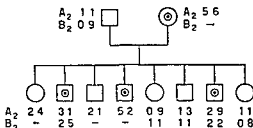


FIG. 1 (Ceppellini) Family showing the enhancing effect of thalassaemia on the levels of A_2 and B_2 (expressed as percentage of the total Hb). Dotted circles haematological signs of *Thalassaemia minima.*

According to the relative electrophoretic mobilities the charge difference between A_2 and B_2 is of the order of that between A and S.

We have up to now identified 29 carriers of B_2 belonging to 4 unrelated families (from our data the frequency of the trait can be estimated at just below one per cent) From 8 matings between a carrier of B_2 and a normal individual (no B_2), 19 normal children and 18 carriers of B_2 have been observed; thus B_2 seems to be inherited as an autosomic, monofactorial, aplosufficient trait, with a segregation of 1 : 1 in this type of mating. We think that B_2 is related to A_2 because in the B_2 carriers (Figs. 1 and 2), the amount of A_2 is decreased to half its normal value, i.e. from 2·5 per cent to 1·2 per cent, while B_2 is present in the same low amount; the sum of the two minor slow-moving fractions adds up to the normal values of A_2. It is of interest that when B_2 is present in a thalassaemia heterozygous carrier, the values of both fractions are doubled and their sum is the high level of A_2 (~ 5 per cent), typical of the thalassaemia trait

(Fig. 1) On this basis it seems reasonable to regard B_2 as a haemoglobin fraction which is allelic to A_2; calling this new haemoglobin locus Hb_2 (the subscript 2 seems suitable for symbolizing the locus which is responsible for the *two* minor fractions) the normal individual is homozygous for the commonest allele and can be symbolized as Hb_2^A/Hb_2^A; the carrier of A_2 and B_2 corresponds to the heterozygous Hb_2^A/Hb_2^B. Finally the homozygous Hb_2^B/Hb_2^B can be anticipated, showing complete absence of A_2 which should be substituted by B_2,

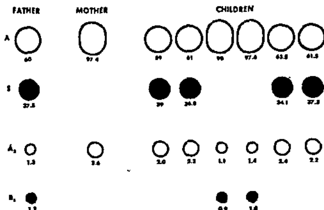


Fig. 2 (Ceppellini). Propositus: the father, carrier of S and B_2 Diagrammatic representation of the electropherograms (pH 8·2—migration from bottom to top—fractions expressed as percentage of the total Hb)

in amounts around 2·5 per cent. Of course only the finding of such a homozygote will definitely establish the relationship of allelism between A_2 and B_2.

As is shown in Fig. 2, a double heterozygous "Hb_1^A/Hb_1^S; Hb_2^A/Hb_2^B" has been found. From the alternative segregation of S and B_2 in the six children it can be suspected that the two loci are linked (the two mutations being in repulsion in the father), but more similar families are needed before it can be accepted as evidence of linkage.

Another collection of pedigrees has some bearing on the present discussion. From the very beginning of haemoglobin studies it has been stated, and generally accepted, that the thalassaemia mutation is independent from the Hb_1 locus (*S* and *C*). In fact, in a few families reported in the literature the two mutations seem to be

transmitted from the same parent to the same child. On the contrary, in our studies we met a number of cases where from a double mutant parent (carrier of sickle cell–thalassaemia disease) mated to a normal subject, each child inherited either one or the other mutation as if they were allelic.

We have made a special search for this kind of family and the results are presented in Table I (Ceppellini, R., Dunn, L. C., and Kunkel H. G., unpublished data)

Table I (Ceppellini)

Matings (STh × Normal)			*Offspring*			
Family code		Ethnic group	S/+	Th/+	STh	Normal
Bac.	*T*	African	4	3	0	0
Man.	*C.*	Greek	2	1	0	0
Ger	*S*	Italian	1	2	0	0
Rag.	*S.*	Afro-Ital.	1	0	0	0
Ree.	*R.*	African	1	2	0	0
X.	*Y*	African	1	3	0	1*
Di.	*D.*	Italian	2	2	0	0
Total observed			12	13	0	1
Exp. for allelism			13	13	0	0
Exp. for independence			6 25	6 25	6 25	6·25

Symbols:

S/+ Sickle-cell carrier (Hb S 30–45 per cent)
Th/+ . *Thalassaemia minima* (microcytosis—A_2 above 3 per cent).
STh : Microdrepanocytosis (Hb S 65–80 per cent—some A present)
Normal : Neither S/+, nor Th/+, nor STh.

* Classified as normal because A_2 low (1 per cent), S absent (but haematologica picture of the thalassaemia or iron deficiency type)

In the absence of an easily recognized specific pigment, the diagnosis of thalassaemia is rather difficult; thus we decided from the beginning to accept as heterozygous thalassaemia carriers (*Thalassaemia minima*) only individuals who show both the typical haematological signs (decreased corpuscular volume, decreased osmotic fragility, anisopoichilocytosis) and an increase of A_2 above three per cent. As sickle cell–thalassaemia carriers only individuals were accepted with the haematological signs of thalassaemia and with a haemoglobin S above 65 per cent (a level which is never

reached by simple S heterozygotes) but with an appreciable amount of Hb A left (to avoid the possibility of misclassifying S–Th and SS homozygotes). In a few cases also the parents of the double heterozygous propositus were examined: 3 were simple Th carriers, one was a simple S carrier. Out of 26 children, 25 show the S/+ or Th/+ phenotype and only one has been classified as normal On the assumption of independence an equal number of children were expected in the 4 classes ($\chi^2 = 21{\cdot}32$ for 3 d.f. $P < \cdot 01$). Therefore we must conclude that thalassaemia (at least the variety with high A_2, here selected) and the S mutations are not independent. Can they be allelic? In family XY a child has been classified as normal, because he had a low level of A_2 (below one per cent) although presenting a haematological picture rather characteristic for microcythemia (microcytosis, decreased osmotic fragility, anisocytosis; however an iron deficiency anaemia was not ruled out); moreover the child born just before this one, was shown to be illegitimate.

If this child is classified as non-thalassaemic the data up to now collected suggest that this thalassaemia locus is distinct from but closely linked with the S locus. In regard to the cases reported in the literature, where the two mutations seem to segregate independently, they can be explained as follows: (1) thalassaemia phenotypes are genetically heterogeneous and may be produced by mutations at more than one locus, one of which is independent from the S locus; (2) these cases are recombinant like (possibly) the child in family XY; (3) diagnostic errors in the absence of electrophoretic analysis and quantitative estimate of A_2.

When these genetic data are used for attempting a phenogenetic interpretation of the mode of action of the Th gene, we meet many difficulties.

It is quite clear that the thalassaemic defect acts to depress more the rate of synthesis of Hb A than the rate of synthesis of other haemoglobins, abnormal (S, C, E) or normal ones (A_2) It has been said that Th^T is a specific modifier of dominance but this explanation is not a satisfactory one. A much simpler explanation would be possible if this mutation could be regarded as an amorph or ipomorph allele of the S locus, impairing the rate of synthesis of Hb A; or still better, accepting the model presented by Dr. Ingram, Th would impair the synthesis of the β peptide: in the Hb^S_β/Hb^T_β heterozygotes the excess of β^S over β^A peptide would result in a relative excess of Hb S over Hb A. As Dr Ingram suggests, a similar defect could also affect the synthesis of the α peptide; thus two genetically independent varieties of thalassaemia, α-type and β-type, could be visualized.

Unfortunately many biochemical data are still lacking; among others (1) it is not known whether the Th defect acts primarily on the synthesis of the haem or on the synthesis of the globin; it is apparent that the two processes are intimately related and directly influence each other [Rimington, C., (1959). *Brit. med. Bull.* 15, 19]; (2) it is not known *if the A fraction which is present in different amounts in Th* homozygotes, is identical, on finger-print analysis, with normal A haemoglobin, or differs for some amino acid.

From what has been said it is clear that only the integration of genetic data with biochemical data allows the best understanding of the gene; for this reason the correct mapping of the *Th* mutation (or mutations) relative to other *Hb* loci, is a problem of the greatest importance.

Itano: Non-specific alterations of the haemoglobin molecule may occur within red cells after synthesis and in samples during preparation, storage and analysis. Therefore I doubt that it will be possible to ascribe each of the minor components of haemoglobin to mutations or to anomalies in chain composition.

Neel I want first to make clear where we stand as regards our GS-thalassaemia family. The evidence that the G mutation involves the amino acid next to the SC change forces one to do some very serious thinking. If what we interpreted as G is actually some strange artifact, or if there should be a "hybrid substance" formed in some persons heterozygous for the thalassaemia and haemoglobin S genes, then we may be forced to a reinterpretation of this pedigree. At the moment the interpretation of this family is unsettled.

Now with reference to the thalassaemia locus, the data shown regarding the allelism of the genes resulting in thalassaemia and haemoglobin S are incontestable. However, there are several other pedigrees which do suggest non-allelism of thalassaemia with S; and also at least two pedigrees suggest non-allelism of thalassaemia with C. We are responsible for one of those pedigrees. We have just restudied it, very carefully, and we think that the evidence for non-allelism stands [Cohen, F., Zuelzer, W. W., Neel, J. V., and Robinson, A. R. (1959). *Blood*, in press]. In my opinion, when all the available material is reviewed, there is evidence for a heterogeneity in the entity we term thalassaemia, one type allelic to or linked with the *S–C* locus, the other(s) segregating independently. Now it is interesting that the family exhibiting non-allelism of thalassaemia with the *S* locus which we studied was a Negro family, and in Negroes with thalassaemia the A_2 component is not nearly as frequently elevated as in Italians. So here is some evidence that a "different" thalassaemia gene tends to occur in Negroes, and this is where the evidence for non-allelism comes in.

Ingram: I would be happy with any number of thalassaemia loci up to two!

Harris: With regard to the hypothesis that Dr. Ingram has put forward, the point as I understand it is that if you have the sickle-cell gene and this kind of thalassaemia gene together, the chances are you will get an extra component I also understand that many examples of sickle-cell thalassaemia disease have been reported. Is there any indication of such a component in all the quite large number of cases which have been described?

Ingram: No, but that has not been looked for.

Lederberg: What about the father of the persons indicated?

Ingram: The father was SA.

Lederberg: This could be another type of recombination product. If this is a T gene which blocks the formation of the α chain it may still be able to form a recognizable haemoglobin in combination with the β chain of haemoglobin S That, I think, is what you mean by a "hybrid substance".

Ingram: This is also a possibility, but I am not sure whether you could do this background for the whole family.

Lederberg: What exactly was the evidence that H is β_4^A?

Ingram. The evidence is partly the type of hybridization experiment which Dr. Itano talked about, compatibility with β_2^A. Also by chemical analysis which shows that all the peptide chains of haemoglobin H begin with val–his–leu which is the beginning of the β chain, and not with val–leu which is the α chain.

Lederberg. When haemoglobin H is dissociated there is no component which will then combine with the β chain?

Ingram: That is correct.

Lederberg: But when it is dissociated it also reassociates?

Ingram: Yes; and also if you take haemoglobin H and haemoglobin S and recombine you get a new haemoglobin which moves close to the normal.

Montalenti: Some electrophoretic patterns of haemoglobins have been obtained in my laboratory by Drs Adinolfi, Chieffi and Siniscalco, in the lamprey, one of the lowest vertebrates [Adinolfi, M., and Chieffi, G. (1958). *Nature (Lond.)*, 182, 730; Adinolfi, M., Chieffi, G., and Siniscalco, M. (1959). *Nature (Lond.)*, in press]. Larval haemoglobins have been discovered also in fishes, both in Teleosts and in Elasmobranchs. Fig. 1 illustrates the difference in the haemoglobin patterns of the brook lamprey *Petromyzon planeri* during the course of development. The diagram shows the four haemoglobin components in this lamprey, two of which are typical of the larval stage and two of the adult stage. Ammocoetes of *P. planeri* can be found throughout the year in the River Sarno near Naples and they always

show the typical larval haemoglobin pattern except during a short period from January to March (during which time most of them undergo metamorphosis and become adults). Many individuals then possess all four haemoglobin components and the other possible

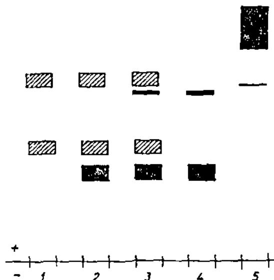


FIG. 1 (Montalenti). Electrophoretic patterns of lamprey haemoglobins (*Petromyzon planeri*) in starch gel at pH 8 8. (Poulik tris-borate system 2 5 v/cm. for 14 hrs.)

Size of the rectangles in the diagram is proportional to the intensity of the Hb bands after staining with benzidine:

(1) typical larval pattern between May and December;

(2, 3) patterns observed between January and March mostly in individuals showing obvious signs of metamorphosis;

(4) typical adult pattern from May onwards;

(5) human haemoglobin pattern in the same experimental conditions.

combinations are of frequent occurrence. In late April and May all animals found in Nature are either of the adult or of the larval type. Later on the sexually mature individuals die after spawning. Only ammocoetes survive and they show, of course, the larval haemoglobin pattern until the beginning of the following winter. From what has been said it is conceivable that the changes of haemoglobin

patterns in the lamprey are of some physiological importance and possibly connected with the morphological changes which take place during metamorphosis and sexual maturation.

Siniscalco: It is worth mentioning, to show that we are not dealing with an electrophoretic artifact, that each of the haemoglobin components when isolated and subjected to a second starch gel electrophoresis preserved unaltered its identity and electrophoretic properties. The multiple pattern of lamprey haemoglobin is also obtainable on starch block and on paper electrophoresis (especially acetate-cellulose paper). It seems therefore quite likely that the separation in different haemoglobin bands is due to differences in the net charge more than to differences in molecular size.

If this is so, and bearing in mind that lamprey haemoglobin seems to consist only of molecules with a single haem, we feel that fine structure analysis might very profitably be used to elucidate more closely this rather complicated situation.

Finally it must be added that there was no obvious genetical polymorphism either in the 119 ammocoetes or the 102 adults so far investigated from the River Sarno, and that exactly the same adult haemoglobin pattern was observed in adult individuals of *P. fluviatilis*, a species closely related to *P. planeri*, which lives in the sea during the larval stage and then comes up to the fresh water to reach maturity.

Brenner: What is the evidence for one haem?

Siniscalco: The molecular weight in the sea lamprey has been measured and is about 16,000 [Wald, G., and Riggs, A. (1951). *J. gen. Physiol.*, **35**, 45; Lenhert, P. G., Lowe, W. E., and Carlson, F. D. (1956). *Biol. Bull.* (*Wood's Hole*), **111**, 293].

Brenner: Have you measured the oxygen equilibrium? Do you get haem-haem interaction?

Siniscalco: No. As I said, we have to go further with the physiochemical investigations. But no haem-haem interaction was found by Wald and Riggs (1951, *loc. cit.*) in the sea lamprey *P. marinus*.

Brenner: This may be very important from the evolutionary point of view, and it may well be important to investigate the two separate components.

Lederberg: Is there some adaptive significance in the shift of haemoglobins?

Siniscalco: This is what we wonder. Evidently there must be something which is connected with the switch from the synthesis of one type to that of the other. All we can say is that when the adult type of haemoglobin appears two other important phenomena occur: individuals undergo metamorphosis, ultimately reaching sexual maturity, and they leave the river bed, where they have been through the whole larval stage, to swim freely in the water.

Itano: We have been interested in lamprey haemoglobin in connexion with the subunits of haemoglobin and the possible evolutionary significance of association–dissociation phenomena. Svedberg has reported molecular weights of 23,100 for *Myxine glutinosa* and 19,100 for *P. fluviatilis*, and has suggested that these haemoglobins were mixtures of monomer and dimer units (Svedberg, T., and Eriksson-Quensel, I.-G. (1934). *J Amer. chem. Soc.*, **56**, 1700). Perhaps the two electrophoretic components of lamprey haemoglobin correspond to the monomer and dimer Svedberg also found that the haemoglobins of reptiles and amphibians, which are four-haem molecules, often have a rapidly sedimenting component which probably corresponds to a doubled molecule (Svedberg, T., and Hedenius, A. (1934) *Biol Bull* (*Wood's Hole*), **66**, 191). Dimerization must therefore be added to the sources of heterogeneity that may occur after synthesis of haemoglobin under genetic control.

Ingram Does lamprey haemoglobin contain any cysteine?

Siniscalco We don't know.

Smithies. With regard to the question of the possible advantage of the haemoglobin molecule containing 4 haems, is this not due to the S-shaped dissociation curve of the circulating haemoglobin permitting more efficient transfer of oxygen to myoglobin? This would be less efficient if you did not have the S-shaped curve which results from interaction of the 4 haems.

Ingram: I don't think that it is the fact that haemoglobin has an S-shaped curve in itself which enables the transfer of oxygen to myoglobin. It is a question of oxygen affinity which is much higher for myoglobin. The sigmoid curve helps in transporting oxygen because it also helps to get rid of waste CO_2, and to take up the maximum amount of oxygen

Monroy: Dr. Siniscalco, have you any evidence as to whether any of your components come from the nucleus?

Siniscalco: No.

Monroy: In our laboratory Dr. D'Amelio has some evidence that in chicken erythrocytes one of the three haemoglobin components present is found in the nucleus This component corresponds to one of the three components which can be identified in the total pattern of haemoglobin. Furthermore, Dr. D'Amelio has analysed serologically these three components and he has found that two out of the three bear the same serological specificity; the third has a different serological specificity. Recently he has been able to identify three components in the haemoglobin of the turtle; and here again only one is to be found in the nucleus. Now the question arises whether the haemoglobin we find in the nucleus is actually synthesized in the nucleus. Recently there have been several reports concerning the

ability of the nucleus to carry out protein synthesis. Dr. D'Amelio has carried out some experiments with isolated nuclei from chicken red cells and reticulocytes incubated in a proper system supplemented with a radioactive precursor. At first, the evidence seemed to point towards an ability of the nucleus to synthesize haemoglobin, but in recent experiments in which the nuclear extract after the incubation was fractionated by means of starch electrophoresis and the different fractions were analysed for both activity and protein distribution, it was found that the haemoglobin extracted from the nucleus did not show any activity, whereas the activity was localized in another protein component.

Short communication:

STUDIES ON FOETAL MYOGLOBIN

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In connexion with the problem of the genetical control of haemoglobin (Hb) synthesis, some studies which were carried out on human and bovine foetal myoglobin (Mb) are reported here.

The existence in Man and in experimental animals of two types of Hb differing in their chemicophysical and functional characteristics, is well known. HbF, present in the foetus and in the newborn, persists for some time after birth and is then gradually substituted by adult Hb. We know that in some pathological conditions (e.g. Cooley's disease) foetal Hb persists in the adult, owing to a genetical error.

Data on the presence and nature of Mb in foetal muscles are scanty and often contradictory. According to the early research by Jonxis (quoted by Millican, 1939), Mb is present in the diaphragm muscle of the newborn but not in the heart muscle, in spite of the fact that the latter muscle is the first to function. Conversely, according to Biörck (1949), during foetal life the human heart contains relatively low amounts of Mb, but at birth the concentration of this pigment is only slightly less than in the adult heart. More recently, Jonxis and Wadman (1952) prepared from the heart of a full-term bovine foetus an extract containing Mb, which they purified according to the method of Theorell and de Duve (1947). In this extract they determined the alkali denaturation time, and the solubility in ammonium sulphate. In view of the results obtained, they admitted the possible existence of a myoglobin of foetal type. Wadman (1954) suggests that in early foetal

life (i.e. before the seventh month, when the Mb concentration in the muscle is still low) a less soluble form of Mb is present.

More recently, Singer, Angelopoulus and Ramot (1955) admitted the existence in the premature foetus and in the newborn of an Mb of foetal type, spectroscopically and electrophoretically different from adult Mb; foetal Mb would be gradually replaced by the adult type pigment during the first six months of life. Timmer, van der Helm and Huisman (1957), working on a preparation from muscular extracts of bovine foetuses purified by ammonium sulphate fractionation and chromatography on Amberlite IRC 50, observed that bovine foetal Mb behaves like adult Mb.

The discrepancy between the above results can be attributed either to the fact that none of the authors used a sufficiently pure, crystalline protein, or to the purification techniques they employed. In fact, methods currently employed for the preparation of adult Mb cannot be directly applied to foetal Mb both because of the very low concentration of MbF in foetal muscles and because of the high solubility in ammonium sulphate of foetal Hb, which is invariably present in large amounts together with foetal Mb. Moreover, no comparative study of the functional properties of the foetal and adult pigments has been carried out so far.

In 1953, in collaboration with Cavallini and De Marco, we isolated foetal Mb from human and bovine heart and, by a suitable modification of the technique we had developed for the preparation of adult type Mb, we obtained it in the crystalline state. The ultraviolet and visible absorption spectra of the two proteins were similar, except for a slight inflexion at 290 mμ in the spectrum of the foetal pigment. Qualitative amino acid analysis of human foetal and adult Hb and Mb by means of paper chromatography (Rossi-Fanelli, Cavallini and De Marco, 1957) revealed that human foetal Mb differs from foetal Hb in that it does not contain cysteine, and from adult Hb in that it contains isoleucine instead of cysteine (isoleucine is not present in adult Hb). Foetal Mb was thus found to be qualitatively similar to ad-

Mb. However, data then available were not sufficient to establish whether or not the two pigments had the same chemical nature.

Recently we resumed the investigation of this problem and studied the electrophoretic behaviour, quantitative amino

Table I

CHEMICAL COMPOSITION OF HUMAN FOETAL AND ADULT CRYSTALLINE HAEMOGLOBIN AND MYOGLOBIN

g amino acid per 100 g protein

	Haemoglobin		*Myoglobin*	
	adult	*foetal*	*adult*	*foetal*
Aspartic acid	9·99	9·59	8·27	8·42
Threonine	6·03	6·98	2·85	2·51
Serine	5·07	6·39	4·43	3·57
Glutamic acid	7·41	6·81	16·17	16·74
Proline	5·02	4·70	3·20	2·26
Glycine	4·28	3·98	6·08	6·47
Alanine	9·83	8·51	5·82	5·43
Valine	11·00	9·34	4·54	4·59
Methionine	1·23	1·84	2·69	2·17
Isoleucine	0·00	1·49	5·00	5·01
Leucine	14·90	13·66	13·12	12·89
Tyrosine	2·90	2·68	2·19	2·22
Phenylalanine	9·62	8·83	6·54	6·37
Histidine	8·49	7·04	7·79	6·90
Lysine	10·64	12·54	15·30	14·95
Arginine	3·48	3·11	2·46	1·86

acid composition, O_2 and CO equilibria, of both foetal and adult Mb. Employing a technique which we have described earlier (Rossi-Fanelli and Antonini, 1956), we showed that foetal human Mb, like the adult pigment, is heterogeneous; both crystalline preparations and muscles contain three components with different electrophoretic mobility. The mobility of the three components of foetal Mb was found to be the same as that of the three components of the adult pigment.

We further investigated the chemical composition of foetal Mb by Moore and Stein's (1951) method for the quantitative determination of amino acids. The results are reported in Table I. Figures for adult myoglobins and for adult and foetal haemoglobins are given for comparison.

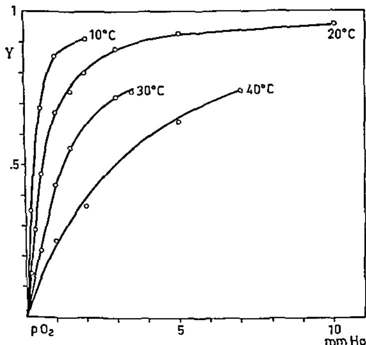
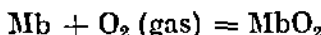


FIG. 1. Oxygen dissociation curves of foetal bovine Mb at different temperatures Tris buffer 0·05 M, pH 7·45, Mb 2×10^{-4} M. Y = fractional saturation with O_2.

These data show that foetal Mb has an amino acid composition practically identical with that of adult Mb, but is markedly different from adult and foetal haemoglobins.

By means of a procedure previously described (Rossi-Fanelli and Antonini, 1958) O_2 equilibrium was comparatively studied in the adult and foetal bovine Mb. O_2 dissociation curves for both pigments at different temperatures are shown

in Figs. 1 and 2. It is evident that the two pigments behave in exactly the same way. The overall heat of the reaction:



$$Mb + O_2\,(gas) = MbO_2$$

$$\text{namely } \Delta H = -\frac{R d \ln K^*}{d(^1/T)}$$

is calculated. The ΔH was found in both cases to be −15 kcal.

FIG. 2. Oxygen dissociation curves of adult bovine Mb at different temperatures. Tris buffer 0·05 M, pH 7·45; Mb 2×10^{-4} M. Y = fractional saturation with O_2.

The partition constants (K) between O_2 and CO for adult and foetal Mb were determined; the values, calculated as described in a previous paper (Rossi-Fanelli and Antonini,

* ΔH is the heat of reaction; K the equilibrium constant; T the absolute temperature; R the gas constant.

1958), are reported in Table II from which it is apparent that even in this respect the two pigments do not differ appreciably.

Table II

RELATIVE AFFINITY OF FOETAL AND ADULT BOVINE MYOGLOBIN FOR O_2 AND CO

$K = (MbCO) \times pO_2/(MbO_2) \times pCO$

Determination	*adult Mb* K	*foetal Mb* K
1	26	28
2	26	31
3	26	28

Temp. 20°, Tris buffer 0 05 M, pH 7 45, Mb 2×10^{-4} M.

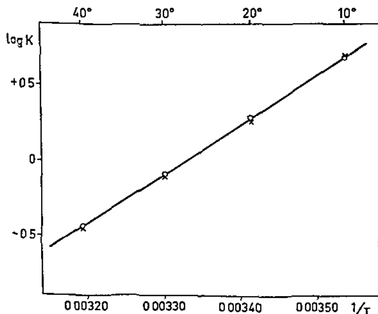


FIG. 3. Influence of temperature on the oxygen equilibrium of foetal bovine (o) and adult bovine (x) Mb.

In conclusion, our experimental results strongly support the identity of the two pigments in several animal species. We

suggest, therefore, that until further investigations reveal some finer structural and functional difference, it is not justifiable to postulate the existence of two different "loci" for the genesis of foetal and adult Mb; on the other hand, it seems legitimate to admit that HbF and HbA_2 derive from two different loci of the same allele. Further evidence for this is provided by recent research where we have shown that HbA_2, isolated by means of starch block electrophoresis, has a different chemical nature from that of adult Hb.

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GENETICS OF THE PLASMA PROTEIN VARIANTS

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The plasma proteins constitute an exceedingly complex mixture of many different protein species. This complexity has been particularly emphasized in recent years with the development of new methods of analysis of protein mixtures which, when applied to human plasma, have revealed how relatively crude were earlier systems of fractionation. When, for instance, plasma is subjected to electrophoresis either in free solution in the classical Tiselius system, or in a supporting medium of starch grains, filter paper or agar, only six components can usually be separated: albumin, alpha 1, alpha 2, beta and gamma globulins, and fibrinogen. If, however, such a separation is combined with electrophoresis in starch gel in a two-dimensional system, about twenty distinct components can be observed. Similarly using the technique of immuno-electrophoresis, where characterization by specific precipitation zones against appropriate antisera is combined with zone electrophoresis, a comparable number of individual components may be recognized. Fractional precipitation methods have also been found to reveal a similar degree of complexity.

In general, it is unlikely that any single technique is capable of a complete resolution of all the individual components present. There are, for example, many enzyme proteins present in such small amounts that they cannot be identified in any general procedure of protein fractionation, though their presence may be readily demonstrated by specific tests based on their enzymic properties. In the same way many of the components of the blood coagulation system are

apparently proteins which are present only in trace amounts, and which can at the moment only be detected by the use of specific clotting tests.

In the last few years a whole series of genetically determined variations in the synthesis of particular plasma protein components in Man have been recognized. They are of obvious significance in connexion with the general problem of the rôle of genes in protein synthesis. Although what is known about them is in many respects incomplete and fragmentary, nevertheless they illustrate rather clearly something of the diversity of phenomena which must be accommodated by any adequate hypothesis relating gene structure to protein synthesis.

Afibrinogenaemia, analbuminaemia and agammaglobulinaemia

In certain instances, individuals with a particular genetical constitution are completely or almost completely deficient in one of the major fractions observed in classical electrophoresis. One such peculiarity is afibrinogenaemia. No fibrinogen can be detected electrophoretically, although immunochemical studies suggest that very small amounts (about 1 mg. per cent) may actually be present (Gitlin and Borges, 1953). The affected individuals suffer from severe haemorrhages and usually die in early life. They appear to be homozygous for a rare mutant gene. There is some evidence that the heterozygotes may have a slightly lower concentration of plasma fibrinogen than do normal people (Frick and McQuarrie, 1954) but this does not lead to any ill effects.

Another example is the condition, analbuminaemia, recently described by Bennhold and co-workers (Bennhold, Peters and Roth, 1954; Bennhold, 1956). In this condition no serum albumin can be detected electrophoretically. Immunochemical studies suggested that some albumin was present but that it amounted to not more than about 1/2000 of the quantity normally found. Curiously enough, the affected individuals suffered remarkably little clinical disturbance from

this and were able to lead virtually normal lives. They are probably homozygous for a rare gene.

Agammaglobulinaemia represents another, though somewhat more complicated, example of the same kind of phenomenon. In the congenital type of this condition little or no gamma globulin is found electrophoretically, and immunochemical methods indicate that in most cases less than about 25 mg. per cent is present (Gitlin, 1955). Besides this, studies by immunoelectrophoresis have shown that at least two proteins which normally form part of the beta fraction and which seem immunochemically distinct from gamma globulin, are also deficient (Gitlin, Hitzig and Janeway, 1956). Affected individuals exhibit a gross defect in the ability to elaborate antibodies. The genetical evidence suggests that, at least in some cases, the abnormality is determined by a rare sex-linked and apparently "recessive" gene.

In each of these conditions the deficiency of the particular protein or group of proteins has been shown to be due to a failure in synthesis and not to an excessive rate of destruction. In fact, in analbuminaemia (Bennhold, 1956) and agammaglobulinaemia (Gitlin, 1957) infusion studies suggest that the rate of destruction of the protein in question may be slightly less than that normally occurring. Another important point is that in none of these cases is there any indication that an unusual kind of protein is formed in place of the deficient one. If such a protein were found in anything approaching the quantities of its normal equivalent, it seems highly unlikely that there would have been any difficulty in recognizing its presence.

Each of these conditions is rare, and one has little hesitation in labelling affected individuals as "abnormal". Other variations in plasma protein synthesis occur, however, in which each of the alternative phenotypes may be found quite commonly among healthy individuals, and these can only be regarded as different versions of so-called "normality". Human populations appear to be polymorphic for the genes responsible.

Haptoglobins

Perhaps the most striking example of such a polymorphism to be discovered so far is the haptoglobin system. Smithies (1955*a* and *b*) showed that when human plasmas were examined

Table I

SEGREGATION OF HAPTOGLOBIN TYPES IN 300 FAMILIES

Type of Mating		*Number of matings*	*Offspring*				
			Haptoglobin types			*No haptoglobins detected*	*Totals*
♂	♀		1–1	2–1	2–2		
1–1	1–1	6	15	0	0	0	15
1–1	2–1	11	15	16	1	0	32
2–1	1–1	10	9	13	0	0	22
1–1	2–2	13	0	38	0	0	38
2–2	1–1	15	2	33	5	0	40
2–1	2–1	48	36	51	34	0	121
2–1	2–2	48	0	61	46	1	108
2–2	2–1	43	1	65	60	2	128
2–2	2–2	30	0	2	75	0	77
1–1	Unknown	3	3	7	0	1	11
Unknown	1–1	9	7	18	0	0	25
2–1	Unknown	18	14	25	17	0	56
Unknown	2–1	18	7	30	9	0	46
2–2	Unknown	8	0	8	25	0	33
Unknown	2–2	20	0	11	24	1	36
Totals		300	109	378	295	6	788

by starch gel electrophoresis, three distinct types of plasma protein pattern might be found. The differences were due to variations in a group of proteins known as the haptoglobins, which in conventional electrophoresis form part of the alpha 2 fraction and which possess the specific property of complexing with haemoglobin. The three types are now referred to as

1–1, 2–1 and 2–2, and in European populations occur with a frequency of about 0·16, 0·48 and 0·36, respectively. Only a single haptoglobin component can be detected in the 1–1 phenotype. A much smaller amount of a component with the same mobility as this is found in the 2–1 phenotype, but none in the 2–2 phenotype. However, both the 2–1 and 2–2 phenotypes show a series of components moving more slowly than the 1–1 component, and it is of some significance that the mobility of each of the components in the 2–1 phenotype is different from that of any of the components in the 2–2 phenotype. Thus, the haptoglobin pattern is qualitatively distinct in the three types of individuals.

Smithies and Walker (1955) suggested that the three haptoglobin phenotypes are determined by a pair of allelic genes, Hp^1 and Hp^2, 1–1 individuals being homozygous for Hp^1, 2–2 individuals homozygous for Hp^2 and 2–1 individuals being heterozygous Hp^1Hp^2. Galatius-Jensen (1958*a*) has reported an extensive body of family data which are in agreement with this interpretation, and the results obtained in our own laboratory on some 300 families, with very few exceptions, also support the hypothesis. Our own data are summarized in Table I.

The hypothesis requires that no 2–2 individuals should occur among the offspring of 1–1 parents, no 1–1 individuals should occur among the offspring of 2–2 parents, and that the matings of 1–1 × 2–2 individuals should result in only 2–1 offspring. There were 11 exceptions to this in our data. Five of these exceptions can probably be attributed to illegitimacy and this interpretation was supported in 3 instances by the blood groups. The remaining 6 atypical segregants all occurred in three closely related families (Fig. 1) and five of the unusual offspring had haptoglobin types incompatible, on the hypothesis, with that of the mother, and so illegitimacy could be excluded. The haptoglobin component, in the exceptional 1–1 parents here, was neither quantitatively nor qualitatively different from what is usually found in the 1–1 phenotype. The exceptional 2–2 children had somewhat

lower concentrations of haptoglobin than are usually found, and the patterns were therefore rather weak. However, they did not appear, with the methods at our disposal, to have any unusual components or to differ in any obvious way from an ordinary 2–2 pattern. We were able to examine further specimens from most of the members of this family about a year after the original ones had been taken. The results were essentially the same as those obtained originally, the only difference being that individual II7, in whom we had been originally unable to detect any haptoglobin at all, proved on

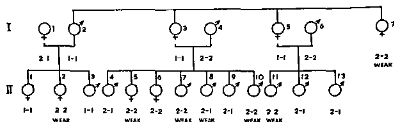


FIG. 1 Pedigree of family B showing atypical segregation of haptoglobin types. (Data from Harris, Robson and Siniscalco, 1958*b*.)

the second occasion to have a weak 2–2 pattern like his anomalous sibs.

This pedigree obviously constitutes a rather exceptional situation and does not override the general validity of the Smithies-Walker hypothesis. It can probably be best explained by postulating that a rare modifying gene or unusual allele was segregating in the family. If, for example, there were an allele Hp^0 such that Hp^1Hp^0 individuals were phenotypically 1–1 and Hp^2Hp^0 individuals were phenotypically 2–2, all the findings except, perhaps, the quantitative differences could be adequately explained.

Excluding the atypical segregants and also the small number of individuals in whom no haptoglobin could be detected, the segregation ratios among the offspring of the different mating types in Table I are in reasonable agreement with the expected Mendelian ratios. Similarly, no significant

deviation from expectation has been observed in any individual family survey so far reported. However, Galatius-Jensen (1958*a*) had pointed out that in the combined published data there is an appreciable deficiency of 2–2 individuals among the offspring of 2–1 × 2–2 matings. Our own results on this mating go in the same direction and *in toto* the effect, though small, appears to be significant ($\chi^2 = 7 \cdot 6$ 1 d.f) (Table II).

Table II

Author	*Offspring of 2–1 × 2–2 matings*		
	Haptoglobin type		*No haptoglobins detected*
	2–1	2–2	
Galatius-Jensen (1958*a*)	104	81	7
Fleischer and Lundevall (1957) and Mäkelä (Quoted by Galatius-Jensen, 1958*a*)	152	124	?
Smithies and Walker (1955)	5	3	0
Harris, Robson and Siniscalco (unpublished data)	126	106	3
Totals	387	314	10

$\chi^2 = 7 \cdot 6$

Galatius-Jensen (1958*a*) has suggested that this phenomenon may be explained by a higher incidence of individuals with no detectable haptoglobin among individuals of the Hp^2Hp^2 genotype than among individuals of the other genotypes. Whether such an effect would be quantitatively sufficient to account for the phenomenon is however uncertain and there is no obvious indication of this effect in the 2–1 × 2–1 matings. Clearly the matter is worthy of further investigation.

The Smithies-Walker hypothesis for the genetics of the haptoglobin types may also be tested by comparing the

observed frequencies of the three phenotypes, in different populations, with the expected frequencies, assuming a Hardy-Weinberg equilibrium. This can be done without making further assumptions only in those populations where virtually

Table III

Incidence of haptoglobin phenotypes in different populations and the expected incidence assuming a Hardy-Weinberg equilibrium

Population		*Total*	*Haptoglobin types*			*Estimated gene frequencies*	
			1–1	2–1	2–2	Hp^1	Hp^2
England	obs.	170	33	88	58	0 43	0 57
	exp		33 1	87 7	58 2		
N Italy (Berra)	obs.	119	20	57	42	0 41	0·59
	exp		20·0	57 6	41 4		
(Cologna)	obs.	208	23	99	86	0 35	0 65
	exp		25 5	94 6	87 9		
Naples	obs.	93	10	44	39	0·34	0·66
	exp		10 8	41 7	40 5		
Sardinia (Illorai)	obs.	147	18	74	55	0 37	0 63
	exp		20·1	68 5	58 4		
Sicily (Catania)	obs.	107	16	53	38	0 40	0 60
	exp		17 1	51 4	38 5		
Borneo	obs.	22	6	10	6	0 50	0 50
	exp.		5 5	11 0	5 5		
Persia	obs.	34	2	13	19	0·25	0·75
	exp.		2 1	12 8	19·1		
Japanese (U S.A)	obs.	23	2	10	11	0·30	0·70
	exp		2 0	9·7	11·3		
Totals	obs.	932	130	448	354		
	exp.		136 2	435·0	360 8		

all the individuals may be classified into one or other of the three types. This calculation is shown in Table III for random samples of a number of populations which we have studied. There is good agreement between the observed and the expected numbers.

Ahaptoglobinaemia

In European populations, occasional individuals are found (less than 1 per cent in most random surveys) in whom no haptoglobins can be detected in the plasma. Whether or not haptoglobins are detected when they are present in very small amounts will, of course, depend on the technical procedures used. However, in most cases which have been referred to as ahaptoglobinaemia or the O phenotype, any haptoglobin present was probably less than 3 or 4 per cent of the quantity usually found. Such a situation could arise because of an excessively rapid rate of removal of haptoglobin from the circulation, or because of defective synthesis. Both these causes probably operate in different cases.

One way in which plasma haptoglobin can be depleted is by haemolysis. Laurell and Nyman (1957) have shown that when haemoglobin is infused into an individual, the haptoglobin-haemoglobin complexes formed are rapidly removed from the circulation and the plasma may be completely depleted of haptoglobin within 24 hours. When the haemoglobin infusion has ceased, fresh haptoglobin begins to appear in the circulation and will reach its original level within a few days. A similar phenomenon happens in haemolytic disease (Nosslin and Nyman, 1958). For example, patients with untreated pernicious anaemia show no haptoglobins, but these appear within a few days of treatment with vitamin B_{12} and reach a normal concentration within 2–3 weeks (Nyman, 1957).

However, there seems no doubt that there also occur individuals with no detectable haptoglobins or with only very minute amounts of them, in the absence of any haemolytic process. This phenomenon appears to be familial. We have encountered one European family in which there were two individuals with no detectable haptoglobin and another individual with only minute amounts. The remaining members of the family had usual concentrations and the segregation appeared to be quite sharp. A similar family has been reported by Sutton and co-workers (1959) and another one by Galatius-Jensen (1958*b*). The pedigrees are shown in Fig. 2.

There seems little doubt that the peculiarity in these families is genetically determined.

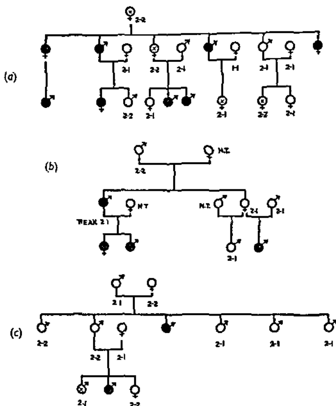


Fig. 2. Pedigrees of families containing cases of ahaptoglobinaemia

(a). Galatius-Jensen, 1958*a*; (b) Sutton *et al.*, 1959; (c) Harris, Robson and Siniscalco (unpublished data).

1-1, 2-1 and 2-2 Haptoglobin phenotypes
● No haptoglobins detected
⊗ Very weak haptoglobins
N.T. Not tested

It is difficult to explain the pedigrees on any hypothesis postulating a further allele at the *Hp* locus because, among other things, ahaptoglobinaemic individuals may evidently

be derived from typical 2–1 × 2–1 matings. The simplest hypothesis is that there is, in European populations, a gene at another locus which can severely depress the rate of haptoglobin synthesis. However, the genetical basis of this phenotype obviously requires much more investigation. The defect can perhaps be regarded as analogous to the conditions, afibrinogenaemia and analbuminaemia, discussed earlier.

Haptoglobins in Negro populations

Most of the previous results concern European or so-called "Caucasian" populations. Studies of Negro populations have

Table IV

HAPTOGLOBIN TYPES IN NEGROES

Population		*Haptoglobin type*				*No haptoglobins detected*	*Total*
		1–1	2–1	2–1 (*Mod*)	2–2		
Gambia	Number	48	23	11	11	64	157
	Incidence	0 31	0 15	0 07	0 07	0·40	
Ibo*	Number	13	12	7	4	34	70
	Incidence	0 19	0·17	0 10	0 06	0·48	
Yoruba*	Number	5	9	4	5	7	30
	Incidence	0 17	0 30	0 13	0 17	0·23	
Negroes from California	Number	21	14	5	10	1	51
	Incidence	0 41	0 27	0 10	0 20	0 02	

* Harris, Lehmann and Robson (unpublished data).

revealed several striking differences. There is often a much higher incidence of the 1–1 phenotype (Sutton *et al.*, 1956, 1959; Allison, Blumberg and ap Rees, 1958); there may be a high incidence of ahaptoglobinaemia (Allison, Blumberg and

ap Rees, 1958); and there is usually a relatively high incidence of a further distinct haptoglobin phenotype, the so-called modified 2–1 (Connell and Smithies, 1959; Giblett, 1959). Our own results on a number of Negro populations illustrate these features (Table IV).

The high incidence of the 1–1 phenotype presumably implies a higher Hp^1 gene frequency than that obtaining in Europe. In contrast to this, what little data we have on Asiatic populations suggest that the Hp^2 gene may be commoner there. The causes of the high incidence of ahaptoglobinaemia in some of these Negro populations are still uncertain. Both genetical and environmental factors could be important. It should be noted that the very high incidence of this phenotype (20–50 per cent) observed in certain populations from Africa has not been found among Negroes in the United States of America where frequencies more of the order of 2–5 per cent have been observed (Giblett, 1959). The high incidence of the modified 2–1 phenotype implies the common occurrence in these populations of yet another genetical determinant which can influence haptoglobin synthesis.

The modified 2–1 phenotype

The modified 2–1 phenotype (Connell and Smithies, 1959) resembles the usual 2–1 phenotype in that the mobilities of the various components present are the same. It differs, however, in the relative concentrations of these components. There is relatively much more of the fast-moving component normally found alone in the 1–1 phenotype, and relatively much less of the more slowly moving components typical of the 2–1 phenotype. The modified 2–1 phenotype is quite clearcut and is readily recognized. It is about one half as frequent as the ordinary 2–1 phenotype in those Negro populations so far studied.

No genetical studies of this phenotype have yet been reported. However, we have collaborated with Dr. Smithies in the investigation of a large Scottish family in which the peculiarity was segregating. The individuals in this family

with the modified 2–1 phenotype are, as far as we know, the only non-Negro group in which it has been observed. The relevant part of the pedigree is shown in Fig. 8.

Two kinds of genetical hypothesis suggest themselves. One would postulate a further allele at the *Hp* locus, the other would postulate a modifying gene at some other locus. If we are dealing with a new allele, then we can ask whether it is in combination with a usual Hp^1 or Hp^2 gene in the modified phenotype. The pedigree indicates that the Hp^1 gene is not peculiar, because in one mating between individuals with a 2–1 modified phenotype and a 2–2 phenotype, two individuals with the usual (i.e. unmodified) 2–1 phenotype occurred

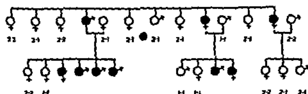


FIG. 8 Pedigree of family containing individuals with the modified 2-1 phenotype.
● Modified 2-1 haptoglobin phenotype
1-1, 2-1 and 2-2 Usual haptoglobin phenotypes.

among the offspring. It should be possible to decide whether one is dealing with a gene at another locus, or a peculiar Hp^2-like allele, by studying a sufficient number of matings of the type 2–1 modified × 1–1. In the one mating of this sort discussed here, there were two modified 2–1 individuals but no ordinary 2–1 individuals in the progeny. Obviously more families are required. For a further discussion see Harris and co-workers (1959).

Another unusual haptoglobin phenotype

Galatius-Jensen (1958*b*) has recently drawn attention to another unusual haptoglobin phenotype which he observed in several members of a single family, and also in another unrelated individual. We have seen a similar phenotype in one individual, and a comparison of the pattern obtained with

this specimen run side by side with a specimen kindly sent to us by Dr. Galatius-Jensen indicated that we were both dealing with the same thing. The phenotype has a series of haptoglobin components some of which have mobilities characteristic of the ordinary 2-1 phenotype and others which have mobilities characteristic of the ordinary 2-2 phenotype. In our specimen, the 2-2 like components appeared to be present in relatively greater concentrations than the 2-1 like components. The pedigrees of the two families in which this phenotype has occurred are shown in Fig. 4. Arguing in the

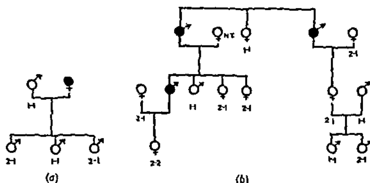


Fig 4. Pedigrees of families containing individuals with an unusual haptoglobin phenotype
(a) Family D17 (Harris, Robson and Siniscalco, unpublished data).
(b) Family II (Galatius-Jensen, 1958*a*)
● Unusual phenotype
N.T. Not tested. 1-1, 2-1, 2-2 Usual haptoglobin phenotypes.

same way as with the modified 2-1 phenotype, we can see that if a new allele at the *Hp* locus is involved, then in the unusual phenotype it must be in combination with a normal Hp^2 allele, because a mating of the unusual phenotype with a typical 1-1 individual gave two 2-1 individuals but none of the unusual phenotype. However, it is clear that other possible modes of genetical causation cannot be excluded.

The transferrins

The routine investigation of human plasma by starch gel electrophoresis revealed another important group of inherited

differences in protein synthesis. The unusual proteins here are variants of transferrin, the iron-binding globulin which forms part of the beta globulin fraction in conventional electrophoresis. Before its identification as transferrin, this component in the starch gel electrophoretic pattern was referred to as beta globulin C. So far, eight different kinds of transferrin have been identified (Smithies, 1957, 1958; Harris, Robson and Siniscalco, 1958*a*; Giblett, Hickman and Smithies, 1959). The nomenclature has been difficult because for a time it was not clear whether or not particular variants discovered by Dr. Smithies and his colleagues in Canada differed electrophoretically from those which we had identified in London. However, for the moment the position has been cleared up and the following terminology is used. The single transferrin present in most people is called C. The three transferrins which move faster than this towards the anode are called B_0, B_1 and B_2, respectively, while the four transferrins moving more slowly than C are called D_0, D_1, D_2 and D_3 (D_3 being the slowest). No more than two of these transferrins have been found in any individual and the various phenotypes which have been identified so far are C, B_0C, B_1C, B_2C, CD_0, CD_1, CD_2, CD_3 and D_1.

The identification of the transferrins may be carried out initially with unidimensional starch gel electrophoresis (Harris, Robson and Siniscalco, 1958*a*) but confirmation is required, by the use of a two-dimensional system and also by demonstration that the presumed transferrin will bind iron (Giblett, Hickman and Smithies, 1959).

The relative mobilities of the different transferrins, one to another, appear to be similar in conventional electrophoresis systems to those in starch gel electrophoresis systems, although the resolution is much sharper in the latter. Thus, differences in charge rather than in molecular size are probably the significant thing.

Family studies suggest that there exists a series of genes each of which determines the formation of a particular transferrin. Individuals with two transferrins appear to be heterozygotes, and individuals with one, homozygotes.

Transferrin C occurs in most people, and the other variants are relatively uncommon. However, they vary quite widely in incidence from population to population. Thus, the phenotype B_2C occurs in about 1 per cent of English and Canadian populations, but has not been reported in Negroes. On the other hand, in certain Negro populations and also in a group from Borneo, an incidence of about 10 per cent of the phenotype CD_1 has been observed. This phenotype (Horsfall and Smithies, 1958) is also common in Australian Aborigines. It has not been reported in Europeans.

So far, no specific association between an unusual transferrin and any particular clinical disorder has been found. In particular, we have failed to find anything peculiar about the transferrin in a few cases diagnosed as haemochromatosis, or in several examples of obscure iron-resistant anaemias. Nevertheless, the search for possible associations of this kind is well worth pursuing.

A "new" plasma protein

In the course of studying the genetics of the haptoglobin and transferrin types, we have recently encountered yet another genetically determined variant in protein synthesis (Frazer, Harris and Robson, 1959). Several individuals, all members of the same family, were found to have a plasma protein component different in its electrophoretic properties from any of those usually observed in human plasma. The unusual protein could be detected by unidimensional starch gel electrophoresis, either in the borate buffer system of Smithies (1955*b*) in which it moved slightly faster than the fast alpha 2 component (Fig. 5), or in the discontinuous buffer system of Poulik (1957) in which it moved slightly faster than transferrin C. In these unidimensional systems, the protein resembled in behaviour certain of the transferrin variants. However, in the two-dimensional electrophoresis it was found to behave quite differently. There, the protein *migrated in the conventional* electrophoretic system at pH 8·6 (with the use of either filter paper or starch grains) with about

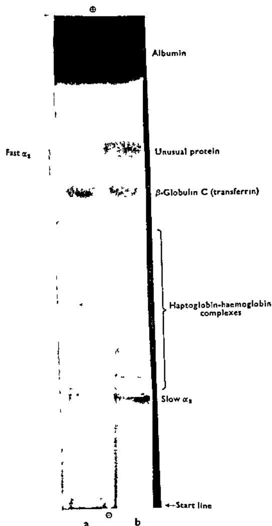
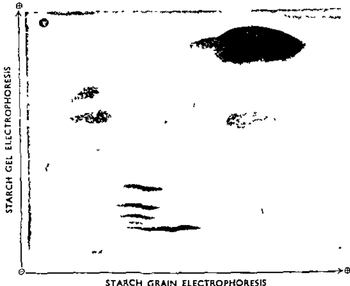


FIG. 5. Electrophoretic patterns obtained in starch gel (borate buffer system)

a. a normal individual; b an individual possessing an unusual protein.



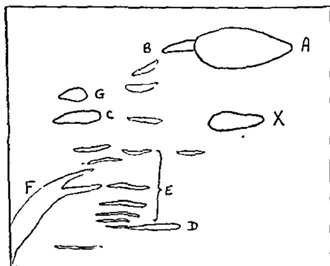


FIG. 6. A two-dimensional separation of the plasma proteins from an individual with the unusual component.

50 µl plasma to which had been added a small amount of haemoglobin was subjected to electrophoresis in starch grains (barbitone buffer pH 8 6) for the first dimension and then in starch gel (Tris/borate discontinuous buffer) for the second *dimension*

Key. A: Albumin; B: Alpha-1 globulin; C: beta-globulin C (transferrin); D: slow alpha-2 globulin; E: haptoglobin-haemoglobin complexes; F: gamma-globulins; G. free haemoglobin; X: unusual component.

the same mobility as serum albumin, and not in the beta globulin fraction as do the transferrins (Fig. 6). Further evidence that it was not a transferrin was provided by the demonstration that it did not bind radioactive iron.

The protein showed another peculiar property: if a red cell haemolysate was added to the plasma, and the plasma-haemolysate mixture was examined unidimensionally in starch gel, the unusual protein gave a marked benzidine reaction similar to that found with the haptoglobin-haemo-

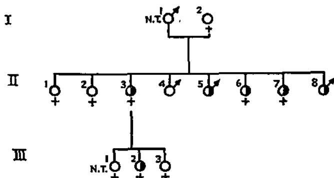


FIG. 7. Pedigree of family showing occurrence of an unusual protein (From Frazer, Harris and Robson, 1959).
◐ Unusual protein present. ○ Unusual protein absent
N.T. Not tested.

globin complexes, although its mobility was not appreciably altered; but when the same plasma-haemolysate mixture was examined two-dimensionally, first by paper or starch grain electrophoresis and then by starch gel electrophoresis, no benzidine reaction could be obtained at all in the region of the unusual component, although the haptoglobin-haemoglobin complexes could be readily demonstrated.

The pedigree of the family in which the protein was observed is shown in Fig. 7. The character segregated as if the individuals who form the protein were heterozygous for a rare gene which is necessary for its synthesis. The peculiarity was not specifically associated with the syndrome of goitre

and deafness which was also segregating in the family, or with either of the haptoglobin types, 1–1 and 2–1, which occurred.

An interesting point is that we have not yet been able to identify any individual component in normal plasma which could plausibly be regarded as the "usual" type of the protein of which the unusual one is a variant. None of the other proteins appeared to be present in obviously diminished quantity. It is possible that such a "normal" equivalent, if it occurs, may move with the serum albumin in starch gel electrophoresis as well as in conventional electrophoresis and thus be obscured. An alternative possibility is that the "new protein" may represent an increased rate of synthesis of a component normally present in only very small amounts.

An albumin variant

Knedel (1957) and Gitlin and co-workers (1958) reported independently what is certainly an inherited variant of serum albumin. This protein occurred in about the same quantity as the normal serum albumin which was also present in the plasma of individuals with the trait. The two together were about equivalent in quantity to the amount of serum albumin normally present. The new component has an appreciably lower electrophoretic mobility at pH 8·6 than normal serum albumin and the two albumins could be readily separated in paper electrophoresis. Family studies indicated in both cases that the individuals with the unusual fraction were heterozygous for a rare gene.

Discussion

This discussion has been confined to the inherited variations in plasma proteins which can be demonstrated electrophoretically. Many other inherited plasma protein variations are known which require other techniques for their demonstration. These mainly concern proteins which are normally present in rather small amounts and whose presence can usually only be detected by testing for certain specific properties which they

may possess. Typical examples are the serum cholinesterase types described by Kalow (1959), the defect in alkaline phosphatase synthesis characteristic of the inherited disease hypophosphatasia, and the abnormality in caeruleoplasmin synthesis occurring in Wilson's disease. One may also mention the various coagulation disorders such as haemophilia, Christmas disease, Owren's disease and so on, each of which appears to have as its basis a deficiency or abnormality of one or other of a number of trace proteins necessary for the normal coagulation of blood.

It is, of course, relatively easy to obtain samples of blood from human beings in order to search for protein differences. Tissue proteins, on the other hand, are far less easily obtained for such studies. It is not surprising, therefore, that most of the inherited differences in protein synthesis of which we are aware, represent peculiarities either in the formation of the plasma proteins or haemoglobin. There is, however, no reason to believe that the multiplicity and diversity of inherited protein differences in plasma are likely to be peculiar to this fluid, and one may expect that many analogous differences in proteins in other tissues occur and will in due course be identified.

At the present time, any discussion of these genetically determined peculiarities in plasma protein synthesis necessarily involves a consideration of how far they can be interpreted in terms of the hypothesis that each gene expresses its effects by specifying the amino-acid sequence in a particular protein or polypeptide chain. In particular, the demonstration by Hunt and Ingram (1959) that haemoglobins A, S and C and other haemoglobin variants differ from one another simply in the substitution of a single amino-acid residue, naturally raises the question of whether analogous differences form the basis of the plasma protein variations.

The situation which perhaps most clearly resembles the one exemplified by the haemoglobins, is that provided by the series of transferrin variants. In both cases, a number of genes occur in human populations each of which results in the

synthesis of a specific variant of a particular protein type. Heterozygotes, where two such genes are present, synthesize two distinct forms of the protein. Although not much is known as yet about the structural peculiarity of the transferrin variants, it seems plausible to suppose that, like the haemoglobins, they may differ from one another by relatively subtle modifications in their amino-acid sequences. The same is also likely to be true of the alternative form of albumin described by Knedel (1957) and by Gitlin and co-workers (1958).

The haptoglobin situation resembles the haemoglobin and transferrin situations in that allelic genes lead to synthesis of different forms of similar proteins. It differs from them, however, in two important respects. In the first place the formation of several distinct protein components appears to be influenced by a single gene difference. Secondly, the heterozygote forms a series of haptoglobin components most of which are qualitatively different from those found in either homozygote. Clearly this poses rather an awkward problem. One attractive hypothesis, which if correct would go a long way to resolve the difficulties, was suggested to us some time ago by Dr. Smithies. In essence the idea is that the series of haptoglobin components present in the 2–2 phenotype may be polymers with increasing molecular weights of a single basic protein or polypeptide unit, whose synthesis is determined by the Hp^2 gene. The single haptoglobin component of the 1–1 phenotype may be a similar basic unit determined by the Hp^1 gene. In the 2–1 phenotype, both units are assumed to combine and form a new polymeric series with molecular sizes different from any of those in the 2–2 phenotype. If something like this happens, and if the two basic units, one determined by the Hp^1 gene and the other by the Hp^2 gene, differ in some relatively simple way in their amino-acid sequence, then the situation may, despite its superficial differences, prove to be closely analogous to that found with the haemoglobins (see also Allison, 1959).

Clearly, however, detailed structural studies on the haptoglobins will be required before these problems are resolved

(see Smithies and Connell, 1959). Among other things, their not inappreciable carbohydrate content will have to be brought into the picture.

Afibrinogenaemia and analbuminaemia present a different kind of problem. Here, the effect of the mutant gene in homozygotes is an almost complete failure in synthesis of a particular protein, and no alternative kind of protein is formed in its place. Several hypotheses can be considered. One possibility is that one is dealing with a chromosomal deletion. This seems unlikely, however, because the defect in synthesis is probably not quite complete and minute amounts of protein are apparently formed. Another possibility is that gene mutation prescribes a new protein structure which is much more unstable, or is formed at a much slower rate, than its normal counterpart and consequently is found in only minute amounts. In this case, the traces of fibrinogen or albumin present in the two conditions might be expected to be structurally different from their normal counterparts even though they behave similarly immunologically. A third hypothesis to explain the situation in analbuminaemia and afibrinogenaemia might be that the protein deficiency may not be a direct consequence of a mutation of a gene which normally determines the structural organization of the particular protein, but may be a secondary consequence of the effects of a mutant gene concerned specifically in determining the structure of some other protein (perhaps an enzyme) which, in turn, is necessary for the synthesis of the protein whose deficiency was directly observed. The underlying defect may be more subtle and less easily detected than its more obvious secondary consequences.

In this connexion, it is worth mentioning that although a particular gene may prescribe the structure of a particular protein and must therefore occur in an individual for that protein to be synthesized, other factors must be important also in deciding whether or not the synthesis of the protein should occur in a given cell at a certain time. Thus, albumin and fibrinogen are thought to be synthesized mainly if not

entirely in liver cells in normal individuals. Yet cells in other tissues must also contain the genes which specify their amino-acid sequences. The metabolic situation in liver cells presumably differs from that in other cells in that it provides the most suitable conditions for proteins to be synthesized in normal individuals. In so far as this metabolic pattern is susceptible to alteration as a result of mutations of genes quite different from those prescribing the structure of albumin or fibrinogen, the rate of synthesis of these two proteins might be markedly altered and, conceivably, almost completely suppressed.

Agammaglobulinaemia provides a similar though probably even more complicated problem. Here, not only is there a gross though not complete deficiency of the series of molecular species which comprise normal gamma globulin, but there has also been demonstrated a deficiency of two other immunochemically distinct protein components. The abnormality is reflected too at the cellular level. Gamma globulins are now generally believed to be synthesized by the plasma cells present in the lymph nodes, bone marrow and other tissues. Good (1955) has shown that both in the lymph nodes and bone marrow in agammaglobulinaemia there is a relative deficiency of plasma cells compared with the other cell types present. The difference is shown up even more strikingly after antigenic stimulation. In normal children, injections of appropriate antigens lead to a marked plasma cell cytosis in the regional lymph nodes. In agammaglobulinaemia, virtually no plasma cell response occurs (Good, 1955).

Whatever the final explanation of these findings in terms of gene action may be, they serve to emphasize the complexity that the problem may assume, even when examined at the level of anomalies in protein synthesis due to single gene substitutions.

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Brenner: Many of these plasma proteins may well not be built of polypeptide chains only. For example, in gamma globulins as shown by Emil Smith, a polysaccharide is attached at a defined place in the amino acid sequence. Presumably this has to be synthesized and it has to be specifically inserted and, we would think, by specific enzyme reactions. This gives you another degree of latitude within the protein; for example, the amino acid sequence may be made but the polysaccharide may be either not made or not attached. The resulting protein may be abnormal; it may even not be secreted from the cell or it may be destroyed in the serum. In other words, in all cases where there appears to be multiple genetic control, one is inclined to predict that further chemical studies of protein will help to clear up the difficulties. One additional point is that in all cases of plasma protein abnormalities turnover studies could be made. In selected cases one could determine whether there is an increase in the rate of destruction of the protein and, if one had the facilities, one could also measure whether there was a decrease in the rate of synthesis.

Harris: As regards the last point it has been done in analbuminaemia, and it was found to be a defect in synthesis. But your other point, I think, is only one part of a more general problem. For instance, some cells make haemoglobin and other cells do not; in the adult some cells make foetal haemoglobin But all these cells, we assume, have the same genetical structure, and have the programme for making haemoglobin of both types. Therefore, there must be in the cell a situation which somehow decides that this cell should be making a particular protein at a particular time. This would depend presumably on some complex metabolic situation which may be determined by a variety of different proteins, and one or other of these being abnormal could alter the rate of synthesis.

Brenner: I would not deny this, but a direct chemical attack on the protein can be useful. At least, if you want to eliminate a direct molecular basis you have the opportunity to do so, and then you can go on to these more complex things.

Smithies: To return to ahaptoglobinaemia, there seems to be a very marked environmental effect here. The data that Dr. Harris showed for Negroes in Africa and Negroes in the United States show a tremendous difference in the frequency of persons with no detectable haptoglobins. Admittedly the populations are not exactly comparable, but in the case of the Negroes in Africa the frequency of ahaptoglobinaemia may be as high as 30 per cent, whilst in individuals with a similar genetical background living in the United States the frequency is of the order of 4 per cent. This suggests a marked environmental effect. Malaria, which can cause haemolysis with con-

sequent removal of haptoglobin from the circulation, is one possible environmental effect, but others (such as liver disease) may be involved.

Dr. Harris, with reference to analbuminaemia and afibrinogenaemia, how good were the antisera used to detect the traces of protein which were thought to be present in the persons described as lacking these proteins genetically? Is it possible that the antisera contained antibodies against some impurity in the original antigen which was responsible for the claimed immunological demonstration of traces of the genetically "absent" proteins?

Harris: I do not know how good the antisera were, but I feel sure that the people who did the work were well aware of the problem. Another interesting point is that Bennhold found, on treating analbuminaemia by injecting normal serum albumin, no apparent antibody response. Bennhold attributed this to the fact that analbuminaemics do, in fact, make a little of their own albumin, and therefore would not produce antibodies against the material injected. I do not know how strong this argument is but perhaps Sir Macfarlane Burnet could comment on it.

Burnet: If any detectable amount of albumin is produced in these cases then you would have a reason for not getting any antialbumin when normal albumin was injected; and equally I would think that that finding rather links with my previous suggestion that if there is initially a complete absence of albumin and then a back mutation produces some, this would be expected to act as an antigen.

BIOCHEMICAL ASPECTS OF THE INHERITED VARIATIONS IN HUMAN SERUM HAPTOGLOBINS AND TRANSFERRINS

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Haptoglobins

VARIATIONS in the serum proteins of normal humans which are genetically controlled have recently been demonstrated by electrophoresis in starch gels. The haemoglobin-binding protein of serum (the haptoglobin of Polonovski and Jayle, 1939) was shown to vary in different individuals by Smithies (1955). A simple genetical hypothesis involving two autosomal alleles (Hp^1 and Hp^2) was suggested by Smithies and Walker (1955, 1956) to account for the inheritance of the three haptoglobin types then known; and, in general, this hypothesis has been well substantiated by more extensive family studies (Galatius-Jensen, 1956, 1957, 1958*a*; Harris, Robson and Siniscalco, 1958*a*, Linnet-Jepson, Galatius-Jensen and Hauge, 1958). Individuals of one of the homozygous types (phenotype Hp 1–1, genotype Hp^1/Hp^1) have a single species of haptoglobin in their serum; homozygotes of the other type (phenotype Hp 2–2, genotype Hp^2/Hp^2) have a series of more than ten haptoglobins; heterozygotes (phenotype Hp 2–1, genotype Hp^2/Hp^1) have haptoglobins different from those of *either* of the homozygotes. The starch-gel electrophoresis pattern obtained with the heterozygous type of haptoglobin differs radically from that obtained with a mixture of the haptoglobins of the two homozygous types.

Three inherited forms of haptoglobin different from the three common types have recently been reported. One of these types [the Hp 2–1 (Mod.) of Connell and Smithies (1959*a*) and of Smithies and Hiller (1959)] is similar to the usual

BORATE GEL

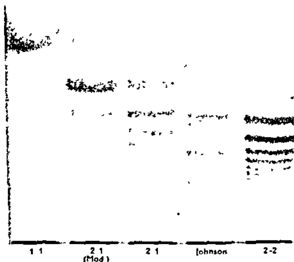


Fig. 1. A comparison of purified haptoglobins of the types (left to right) 1-1, 2-1 (Mod.), 2-1, Johnson type, 2-2. Electrophoresis was carried out in a starch gel, pH 8.5, made with dilute borate buffer, using the procedure described by Smithies (1959). The proteins in the gel were stained with Amido-Black 10B

heterozygous type 2–1, in that the rate of migration in the starch gels of the various haptoglobin components of the two types are equivalent. However, the faster migrating components are present in relatively greater amounts in the modified type. This type is relatively common in American Negroes [approximately 1 in 10, (Giblett, 1959*a*)] but is rare in Whites (Smithies and Hiller, 1959). A second rare type of haptoglobin has recently been observed by Giblett (1959*b*) in the sera of an American Negro female (Mrs. B. Johnson) and her daughter. A complex series of haptoglobin zones, some of which do not migrate at the same rate as any of the previously described haptoglobins, are detectable in the gels with sera of the Johnson haptoglobin type. A third inherited rare type has recently been observed by Galatius-Jensen (1958*b*) in which haptoglobin zones are detectable equivalent in starch-gel mobility to those of *both* types 2–1 and 2–2.

The present writers have recently described (Connell and Smithies, 1959*a*) a simple adsorption and elution technique for isolating haptoglobins from serum in high purity and with good yields. The purified haptoglobins are indistinguishable in starch-gel electrophoretic behaviour from the haptoglobins in whole serum, and completely retain their haemoglobin-binding properties. Fig. 1 shows a comparison of purified haptoglobins of all types so far described (with the exception of the variant described by Galatius-Jensen).

The purified material has been examined (*a*) in order to establish that the various haptoglobin zones demonstrated in the starch gels represent "real" protein components and are not artifacts introduced by the electrophoretic system, and (*b*) to attempt to determine the differences in the genetical forms of haptoglobin at a molecular level.

The electrophoretic separations obtained in starch gels differ in many respects from those obtained in other electrophoretic systems (Poulik and Smithies, 1958), but these differences are now understood and artifacts do not appear to be introduced by the use of the gels. In the case of the haptoglobins the characteristic patterns are obtained in a variety

of buffers, at widely different pH's, and at several ionic strengths. However, the experiments of Franglen and Gosselin (1958), in which metastable polymers of a dye were separated by starch-gel electrophoresis, make it necessary to demonstrate that the many haptoglobins observed in some sera (e.g. type 2–2) are not due to the formation of metastable polymers by this type of haptoglobin. This possibility has been excluded by preparing from the usual starch gels several of the separated haptoglobin zones and showing that their electrophoretic properties are unchanged after storage at 0° or 37°. No reversion to multiple zones occurs. Furthermore, the characteristic patterns of the three common types are obtained under conditions likely to dissociate ionic and hydrogen bonds, namely in gels made with dilute HCl (pH 1·7 and 2·1) and with 8 M urea (see below). Consequently, metastable polymers due to ionic or hydrogen bonds are unlikely to be involved. The presence of 0·01 M versene in the gels is also without effect on the patterns. The many haptoglobin zones observed under the usual conditions are therefore probably distinct protein components. Genetical evidence supports this conclusion. Thus, as described above, modified haptoglobin types have recently been found in which both the relative *amounts* and the relative *mobilities* of the multiple haptoglobins differ from those of the common types—a finding which strongly suggests that all the haptoglobin zones represent "real" protein components.

Studies on the physical nature of the differences in the haptoglobins, and so of the nature of gene action in the haptoglobin system, are still incomplete, but we can report several observations of our own and review some made by other workers which bear on the problem. The one- and two-dimensional starch-gel electrophoretic behaviour of haptoglobin of type 1–1 suggests that it is a single molecular species. Preliminary ultracentrifugal studies (Connell and Smithies, 1959*b*) on the purified protein confirm this. Bearn and Franklin (1958) reached the same conclusion from ultracentrifugal studies with purified type 1–1 haptoglobin com-

plexed with haemoglobin C. On the other hand, the starch-gel electrophoresis results with type 2–2 haptoglobin indicate that this material contains a whole series of proteins. Two-dimensional electrophoretic experiments show that all the type 2–2 haptoglobins migrate at the same rate during filter-paper electrophoresis, although they have widely different starch-gel mobilities. This suggests that the type 2–2 haptoglobins differ from each other in molecular size (see Poulik and Smithies, 1958); the larger molecular species must also have larger net charges or the type 2–2 haptoglobins could not have identical mobilities in the filter-paper electrophoretic system. A simple hypothesis consistent with these observations is that the haptoglobins of type 2–2 include a series of polymers (n=1, 2, 3, 4, etc.) varying in amounts inversely with the degree of polymerization. This hypothesis which could account for the relative mobilities in the gels of the components of type 2–2 haptoglobin is supported by our ultracentrifugal studies with the purified protein. During ultracentrifugal sedimentation a markedly asymmetrical boundary is formed which suggests the presence of a series of proteins of increasing molecular size in amounts which decrease in parallel with the increase in size (Fig. 2*a* and *b*). Bearn and Franklin (1958) obtained the same type of ultracentrifuge pattern with type 2–2 haptoglobin complexed with haemoglobin.

The differences in the three common haptoglobin types are unusually complex to be associated with such a simple pattern of inheritance. We observe not only a change from a single protein to a series of polymers in going from one homozygous type (1–1) to the other (2–2), but also in the heterozygotes (2–1) a pattern is obtained which is quite unlike a mixture of the two homozygous types. We have felt for some time that this complex situation might be the result of a relatively *minor* molecular difference between the haptoglobin produced under the influence of the gene Hp^2 and that produced under the influence of the gene Hp^1, such that the complex series of proteins are formed only in the presence of haptoglobins related to the gene Hp^2. A relatively small alteration in a

protein, such as the introduction of a cysteine residue into the molecule, might permit the formation of a series of polymers which could not be formed by the unaltered protein. We have therefore attempted to break down the haptoglobins to

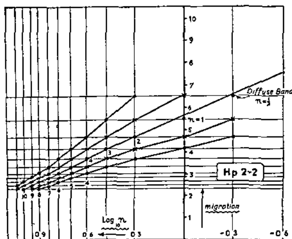


FIG. 2*a* Migration versus log *n* (zone number) for type 2–2 haptoglobins

The distance (in arbitrary units) of migration in a starch gel of the individual protein zones of a purified preparation of type 2–2 haptoglobin (cf. Fig. 1) is plotted against the logarithm of the zone number. The zones are numbered consecutively in order of decreasing mobility *and the number* 1 *is assigned to a different* zone for each of the five curves plotted. A straight line is obtained when the number 1 is assigned to the fastest migrating heavily staining zone, and 2, 3, 4 etc. to the successive zones of decreasing mobilities. This suggests that these haptoglobins are a series of polymers ($n = 1, 2, 3, 4$, etc.). Note that the migration distance of the fastest (diffuse) haptoglobin zone corresponds to $n = \frac{1}{2}$; possibly it is a "hemimer".

simpler structures in order to detect more readily the basic similarities and differences in the various types. The results of these attempts are the most exciting new developments we have to report here—although they are so recent that we cannot yet draw many conclusions from them.

Since we had been considering the possibility of polymer formation through disulphide bonds, we first attempted to break down the haptoglobins by reduction with 0·01 M thioglycollate (in borate buffer at pH 8·5). We were surprised to find (Fig. 3) completely normal patterns with the purified haptoglobins under these conditions (cf. Fig. 1).

We then considered the possibility that hydrogen bonding was responsible for the stability of the postulated haptoglobin

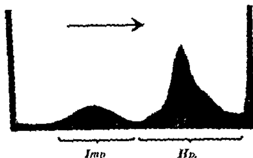


FIG 2b. Sedimentation diagram of a 1 per cent solution in 0 1 M-NaCl of purified type 2-2 haptoglobin 80 min. after reaching 59,780 r.p m in a Spinco ultracentrifuge at approximately 21 5°. The boundaries corresponding to the haptoglobins and impurities are indicated.

polymers and examined the proteins in 8 M urea—a reagent known to disrupt intramolecular hydrogen bonds. The results of an experiment with an 8 M urea/borate gel are shown in Fig. 4. Once again the patterns are essentially indistinguishable from the usual borate patterns. However if gels containing both thioglycollate and 8 M urea are used the haptoglobin patterns are completely changed (Fig. 5). The haptoglobin polymers are broken down (probably as a result of the cleavage of disulphide bonds made accessible to the thioglycollate by the 8 M urea) and the patterns become more simple. We can begin to see that the three types differ in a somewhat less complex manner than is suggested by their usual starch-gel electrophoretic behaviour. The product of

this cleavage treatment applied to type 1-1 haptoglobin migrates essentially as one component (with a greater mobility however than that of the untreated haptoglobin). The multiple components of the type 2-2 haptoglobin are completely broken down by the treatment with the formation of one (the major) cleavage product having the same mobility as the type 1-1 cleavage product together with at least one component of greater mobility. In contrast to the results obtained with the untreated haptoglobins, the heterozygous type now appears to be intermediate between the two homozygous types, and is closely similar to a mixture of them.

We conclude from these preliminary experiments that the three common haptoglobin types (and, see below, the modified types) have in their structure disulphide bonds which are necessary for the integrity of the proteins and for polymer formation, although we cannot be certain that the monomers are actually linked together by disulphide bonds. Nevertheless, these experiments throw considerable light on the nature of gene action in the haptoglobins. There appears to be a major part of the protein molecule common to all the haptoglobin types. When genes other than Hp^1 are present then additional structural elements are involved in the haptoglobin molecules. The nature of these additional elements is as yet unknown, but they do not appear to be the same in all the complex types. A comparison of all the types available to us illustrates this. In Fig. 6 is shown the result of comparing in a urea/thioglycollate gel the same samples (5 types) as were used for the urea experiment illustrated in Fig. 4. The faster migrating cleavage products in the several types differ, and the differences appear to be considerably greater than could be introduced accidentally by the presence of impurities in the preparations (see Fig. 4 for comparison of the purity of these same preparations). The differences between the type 1-1, Johnson type, and type 2-2 haptoglobins are particularly marked. Experiments of this type, although at present quite crude, indicate a new approach to solving the problem of gene action in the haptoglobins, for they will probably enable us

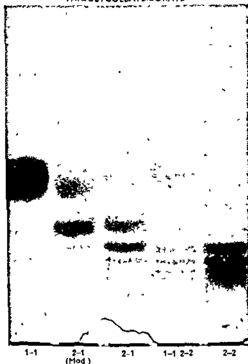


FIG 3 Starch-gel electrophoresis of haptoglobins in the presence of thioglycollate The gel contained 0 01 M thioglycollate and dilute borate buffer The samples are (left to right) purified haptoglobins of types 1–1, 2–1 (Mod), 2–1, a 50/50 mixture of 1–1 and 2–2, 2–2

to separate the variable portion of the different genetical forms of haptoglobin and hence determine the nature of the variations.

Transferrins

The presence of a β-globulin (D) not previously observed was detected by one of us (Smithies, 1957) in the sera of 2 (out of 50) New York Negroes and 5 (out of 23) Australian Aborigines. This β-globulin was present in approximately the same amount as, but migrated less rapidly than β-globulin C (Poulik and Smithies, 1958) which is quantitatively the chief β-globulin in most sera. The presence of the β-globulins C and D in the serum of an individual was shown by Horsfall and Smithies (1958) to be under simple genetical control by two autosomal alleles. Sera from homozygotes contain either β-globulin C or D; sera from heterozygotes contain both β-globulins in approximately equal amounts. A mixture of sera of the two homozygous types is indistinguishable with respect to the β-globulins from serum taken from a heterozygote. Smithies (1958) observed a further β-globulin variant (B) in 4 (out of 425) Canadian Whites, and suggested as a result of preliminary family studies that a third allele was possible at the β-globulin locus. Harris, Robson and Siniscalco (1958*b*) observed two other variants and quoted the pedigree of a family to illustrate the inheritance of one of them.

The identity of the variable β-globulins was discussed by Smithies and Hiller (1959) who concluded that they were iron-binding proteins (transferrins). Direct proof of this was obtained by Giblett, Hickman and Smithies (1959) from experiments with radioactive iron.

At present 8 transferrins (B_0, B_1, B_2, C, D_0, D_1, D_2 and D_3) have been recognized in man. Giblett, Hickman and Smithies (1959) note that the transferrins (B_0, B_1 and B_2) which migrate faster than the common transferrin C are most frequently observed in Whites, those (D_0, D_1, D_2 and D_3) migrating more slowly occur most often in Negroes. The limited family studies reported suggest that persons with a single transferrin

are homozygotes, and those with two are heterozygotes. No individuals have been observed with more than 2 transferrins. There is no evidence as to whether or not all the inherited forms of transferrin are controlled at a single locus, since no population has been studied in which more than one transferrin variant occurs frequently.

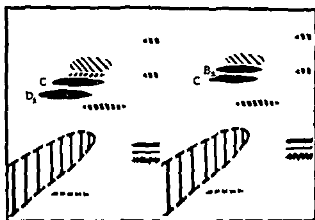


FIG. 7. A diagramatic representation of the two-dimensional filter-paper and starch-gel electrophoretic behaviour of the transferrins B_2, C and D_1 in the sera of two heterozygous individuals. The horizontal direction in the figure corresponds to the filter-paper electrophoresis, the vertical to the starch-gel electrophoresis. The transferrins (B_2, C and D_1) are indicated in the figure.

Some evidence is available (see Smithies and Hiller, 1959) on the nature of the molecular difference between the genetical forms of transferrin. Two-dimensional starch-gel electrophoresis experiments (Fig. 7) indicate that the transferrins B_2, C and D_1 differ in their electrophoretic mobilities both in the filter-paper electrophoretic system (the horizontal direction in the figure) and in the starch-gel system (the vertical direction in the figure). This suggests, but does not prove, that these transferrins, and probably the other transferrins also, differ with respect to charge rather than molecular size.

If this is the case then there are at least 8 possible forms of transferrin, each carrying a different net charge. Any one amino-acid side-chain can have three charged forms: positive, zero or negative. If only one amino acid were interchangeable in the different genetical forms of transferrin, then only three transferrins would be detectable by electrophoresis—a situation similar to that discussed by Hunt and Ingram (1959) for the haemoglobins C, S and A. So we must conclude that several amino acids are interchangeable in the transferrins if the 8 forms so far described differ only with respect to net charge.

The detection of differences in proteins by electrophoretic methods depends on either differences in charge and/or molecular dimensions in the protein molecules. The genetical forms of haptoglobin probably differ in size, and the transferrins in charge; consequently, we are able to demonstrate polymorphism in the respective proteins. Perhaps the detection of polymorphism in these systems reflects nothing unusual in the proteins concerned, but rather that they differ in a way which we can detect. Thus, although the distinguishable transferrins probably differ in charge there may well be other indistinguishable forms of transferrin which do not (e.g. no charge difference would follow the replacement of a valine residue by an isoleucine residue). Such polymorphism may be very difficult to detect if the change in biological function of the protein involved is small.

Summary

The genetically controlled forms of haptoglobin (the haemoglobin-binding protein of serum) have been purified by a simple adsorption and elution technique. Electrophoretic and ultracentrifugal studies suggest that the haptoglobin of one homozygous type consists of a single molecular species. Haptoglobin of the other homozygous type probably includes a series of polymers. The heterozygous type differs from a mixture of the two homozygous types. Thioglycollate and 8 M urea alone are without effect on haptoglobins. Together these reagents cleave the haptoglobins and completely break down

the polymers. Starch-gel electrophoretic studies of the products of the reductive cleavage suggest that a major part of the molecule is common to all genetical types, but that faster migrating portions of the cleaved proteins differ in the several genetical forms. The nature of gene action in the haptoglobin system may be solved by further studies of these cleavage products.

Seven serum β-globulin variants (B_0, B_1, B_2, D_0, D_1, D_2 and D_3) which are under genetical control have been described in addition to the common β-globulin C. The variable β-globulins have been identified as transferrins by their property of binding iron ^{59}Fe. Family studies suggest that sera from homozygotes contain a single transferrin, and sera from heterozygotes contain two transferrins. Electrophoretic experiments indicate that the genetical forms of transferrin may differ in net charge. Several amino-acid residues must be variable in transferrin to account for the occurrence of 8 forms of transferrin if these proteins differ only with respect to net charge.

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DISCUSSION

Brenner: How much of these haptoglobins can you isolate?

Smithies: They are comparatively easy to purify. Our method is a single-step procedure which enables haptoglobins to be prepared in about 40 per cent yield from serum, with a purity of the order of 70–90 per cent (Connell and Smithies, 1959*a*, *loc. cit.*). Approximately 40 mg. of haptoglobin can therefore be obtained from 100 ml. of serum.

Brenner: One suggestion can be made immediately which would really provide evidence for your theory. After you have reduced the protein you could react it with radioactive iodoacetamide which will block the —SH groups adding carbomethyl residues. If you then finger-printed the resulting mixture you could ignore the rest of the protein and look directly for altered cysteine-containing peptides, particularly for new cysteine residues.

Smithies: We hope to do something like that. The results I showed with urea and thioglycollic acid are quite recent, so that at present we have not really had time to do much more than map out the way for future work. The separate urea and thioglycollic acid experiments were made a year ago, but only recently did we try both reagents at once (following a discussion with Dr. R. L. Baldwin in Wisconsin).

Brenner: My second point is a general one. I am not sure that it can be said that all changes in amino acids affecting charge will give the same charge difference on the protein, irrespective of the position of the amino acid. The p*K* of internal amino groups varies quite considerably. I don't think it is necesssary to postulate double amino acid changes.

Smithies: I agree with your general point that a change in an amino acid residue might alter the p*K* of a neighbouring residue. However, since there are at least 8 different electrophoretic forms of transferrin I would be surprised if the number of amino acid residues which vary in these proteins was not greater than one.

Brenner: Is it true that in haemoglobin E one has evidence for this fact?

Ingram: The whole subject of electrophoretic changes which can be ascribed to amino acid constitution is unclear at the moment, but all possibilities should be considered. If you change the charged amino acids you get the corresponding electrophoretic change for the whole protein. You can also postulate that if you change certain uncharged amino acids only the structure will be altered in such a way that also you get electrophoretic differences due to covering or uncovering charged groups or altering their pK's through changes in their environment. As regards haemoglobin E, it is safe to say that we cannot completely correlate the observed amino acid charge difference with the behaviour of the intact protein. I personally think there are two effects, a primary effect of amino acid substitution and secondary effect in which this substitution has altered the architecture of the protein.

Smithies: Yes, I agree with this completely. It is just a generalization that if one regards the electrophoretic differences as due primarily to electric charge, then one has to assume that at least 4 amino acid residues must be variable in the transferrins. However, if there are secondary effects then this will reduce the number of amino acids which must be presumed to vary.

Lederberg: Dr. Ingram, do you think it worth while to induce further electrophoretic differences, for example by removing free amino groups with nitrous acid?

Ingram: No, I don't think so, and one of the reasons is that such chemical alterations are not quantitative, not clean enough, although in theory it is a good idea.

Brenner: We have gone into this in an attempt to find a more efficient screening method for proteins, and it seems that it can work only when there is a change in a cysteine; in this case one can really put on a charged group. One might possibly do things with serine or threonine, but the reactions are not clean. Of course, a vast number of so far undetectable changes in proteins, and which we feel could not be detected even on finger-printing, are those involving the non-polar amino acids. This presents one of the big problems of screening the molecular structure of proteins. There are only rather tedious ways around this difficulty, and it seems that one may be compelled in many cases to go even further than finger-print screening and actually determine amino acid compositions for all the peptides. This would be too much to do routinely, but automatic methods are being developed and it may soon be technically feasible.

Lederberg: Dr. Smithies, do the different genetic forms of haptoglobins have the same isoelectric point?

Smithies: Yes, I believe that they do although I know of no exact measurements to prove this. The purification procedure we have

used for all the types employs a positively charged ion-exchange resin at a pH at which the haptoglobins are kept negatively charged, whilst most of the other serum proteins are positively charged, so that the negatively charged haptoglobins are more or less selectively adsorbed to the resin. At pH 4·2 all the types of haptoglobin can be prepared in this way, but at pH 3·9 none are obtainable. This suggests that the variation, if any, in isoelectric point between the different types is quite small.

Ingram: How important is the use of a borate buffer?

Smithies: Hardly important at all, because we can duplicate all these results in phosphate, veronal and other buffers.

Ingram: Is there any suggestion that you have carbohydrate components in the haptoglobins?

Smithies: Yes there is. Jayle and Boussier have established clearly that there is quite a high carbohydrate content in haptoglobin [Jayle, M. F., and Boussier, G. (1954). *Bull. Soc. chim. Biol.*, 36, 959].

Brenner: What is the molecular weight of the type 1–1 haptoglobin?

Smithies: D. B. Smith in Ottawa has done ultracentrifugal studies (unpublished) on the purified haptoglobins, using the approach to equilibrium method, and he finds a molecular weight of about 100,000 for the type 1–1; for the type 2–2 the average molecular weight is about 400,000 and for the type 2–1 the average molecular weight is about 220,000. This is the order of sizes expected from the starch-gel electrophoretic behaviour of the several types of haptoglobin.

Ingram: I was intrigued by your picture of reduced haptoglobins which showed two components for the 2–2 and for the heterozygote and only one component for the 1–1. Are you suggesting that in the case of a 2–2 type there is a completely new protein component with fast migration, or do you think this is a modification of a slower one, and that there are really two components superimposed in the reduced type 1–1?

Smithies: I cannot hazard a valuable guess at this stage of the work. Electrophoretically the cleavage product of type 1–1 haptoglobin appears to be essentially a single component, and an additional component appears in the cleavage products of the types 2–1 and 2–2, but I suspect that when we improve our methods the situation will prove to be more complex.

Morgan: Dr. Smithies, have you studied the carbohydrate components in your purest haptoglobin preparations? Is there a different sugar in each haptoglobin type?

Smithies: We have not done any work on the sugars.

Cavalli-Sforza: Dr. Smithies, is there information available on which substances other than haemoglobin have an affinity for haptoglobin? Is it the globin part of the haemoglobin that has an affinity for haptoglobin?

Smithies: I'm not sure that anybody has prepared globin from haemoglobin and then established that it combines with haptoglobin, but it is my impression that it is the globin part of the haemoglobin which is combining. As for other materials which have an affinity, it has been suggested that vitamin B_{12} is bound by the haptoglobins.

Ingram: Are the haptoglobins able to distinguish between the series of abnormal haemoglobins and also between haemoglobins of different species?

Smithies: I don't know of any test of this although in Detroit M. D. Poulik at one time planned to try the abnormal haemoglobins. Human haemoglobin will combine with rabbit haptoglobin, and rabbit haemoglobin with human haptoglobin, so I think that any such distinction between normal and abnormal haemoglobins by the haptoglobins is unlikely.

Lederberg: Is it known that haptoglobin does *not* contain the same α or β chains as haemoglobin?

Smithies: I don't think it is known. This might be rather interesting, because perhaps haptoglobin can combine with haemoglobin since it does in fact contain the same amino acid sequence as the α or β chain.

Brenner: You then have to postulate that in the presence of haptoglobin the haemoglobin dissociates.

Smithies: I think it will dissociate, because (although we believe that haptoglobin type 1–1 normally combines with one molecule of haemoglobin) when insufficient haemoglobin is added (i.e. less than will saturate the haptoglobin) you get an intermediate complex which migrates in starch gels more slowly than uncombined haptoglobin but faster than fully saturated haptoglobin (Laurell, C. B. (1959). *Clin. chim. Acta*, **4**, 79).

This suggests that haptoglobin can pick up a half-molecule of haemoglobin (by dissociating haemoglobin) when there is insufficient haemoglobin to saturate all the haptoglobin.

Ingram: It is known that globin, which is a half-molecule, does polymerize; it forms polymers and precipitates perhaps a series of them as in the haptoglobins. But one would then expect that haptoglobin should combine with haem.

Smithies: Is it known whether the part of haemoglobin that causes the α and β chains of the haemoglobin to combine together is the same part that combines with haem?

Ingram: That is not known.

Smithies: Then it would not necessarily follow that haptoglobin would have to combine with haem.

Itano: It is doubtful that haem is the site at which haptoglobin is bound to haemoglobin. Large ligands are bound to haem with difficulty and tend to denature haemoglobin after they are bound. The suggestion that haptoglobin is the same as globin can be tested by adding less than an equivalent amount of haem to globin and looking for a molecule that has the properties of a haemoglobin-haptoglobin complex. As for the proposal that one of the complexes is composed of haptoglobin and a half-molecule of haemoglobin, the presence of only one such complex suggests either that this particular half-molecule is a product of symmetrical dissociation or that only one of the two products of asymmetric dissociation is capable of associating with haptoglobin

Lederberg: What is known of the haptoglobin in the individuals who are homozygous for abnormal haemoglobin; have they been looked at?

Smithies. I don't know what they are like or, indeed, if they have been examined.

SOME IMMUNOCHEMICAL ASPECTS OF THE PRODUCTS OF THE HUMAN BLOOD GROUP GENES

W. T. J. MORGAN

The Lister Institute of Preventive Medicine, London

ONE of the purposes of this symposium is to collect, discuss and summarize information which will help us to understand how the products of the action of closely related genes differ and to appreciate more completely the influence of genes on each other in biochemical terms. The present writer's contribution to the subject will be to consider what we have come to know about the molecular basis of inheritance in Man through a study of a normal inborn variation, that of blood group specificity. The blood group specific character of erythrocytes and secretions does not vary during life, except perhaps under abnormal circumstances, and since the specificity has been thoroughly studied by serologists and the inheritance of the characters by geneticists, it seems that the substances giving rise to group specificity offer a useful and interesting field of investigation for the biochemist. The results obtained by all three methods of approach, immunological, chemical and genetical, will allow gene controlled and inherited characters to be associated with unique chemical structures and thus contribute to our understanding of chemical individuality in Man.

General observations on the isolation and purification of the group specific substances

In attempting to correlate changes in chemical structure with genetical pattern in the field of human blood groups, it is essential to consider carefully the origin of the materials to be studied. As early as 1947 it was emphasized (Morgan, 1947)

that pooled secretions obtained from many individuals were not particularly suitable starting materials but, where such a source is unavoidable in order to procure a larger quantity of substance for subsequent chemical studies, care should be taken to match the blood phenotype and secretor status of the individuals supplying the secretion. The materials we have studied have been obtained almost exclusively from individual donors and this has been achieved by utilizing selected ovarian cyst fluids which contain a relatively large quantity of specific blood group substance produced by one individual. In this way sufficient material has been obtained from a single source to allow of its isolation and thorough purification. Each preparation of specific substance finally obtained requires an examination to determine the extent to which heterogeneous material is present. Freedom from minor amounts of contamination is probably impossible to prove with our present techniques for the resolution of mixtures of neutral mucopolysaccharides, such as are encountered in native secretions. The most favourable results of physical and chemical measurements, involving ultracentrifugation, electrophoresis, fractional solubility tests, etc., when considered individually or together are able to give only a limited assurance for lack of heterogeneity. It has been indicated that the method of specific serological precipitation allows an absolute degree of purity to be established but it now seems possible that even here the conclusions which can be reached, concerning homogeneity of a material, are more restricted than was originally believed. Results obtained from all three kinds of measurements—physical, chemical and serological—on the other hand, give ground for more confidence in assessing freedom of the material from contamination with other substances.

When the materials are finally considered satisfactory on the basis of all the above examinations, it is of importance to collect as much evidence as possible that the specific substances recovered are unchanged from the form in which they exist in the native secretions. Obviously, if a material is

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warmed to about 60°, a precipitate formed which contained highly purified specific blood group substance closely similar in properties to the substance isolated from a portion of the same secretion by extraction with 90 per cent phenol. The procedure could be applied directly to native secretions, but it was found that the yield of specific substance was much lower than if a partial purification had previously been carried out.

A preliminary examination of the products extracted from dried, crude or partially purified blood group substances by dimethylsulphoxide has also been made but the procedure, as developed so far, is not entirely satisfactory. It has nevertheless given small amounts of specific blood group substances which are closely similar in physicochemical properties to the specific substances obtained from the same starting material by extraction with 90 per cent phenol or treatment with hot (60–70°) saturated ammonium sulphate.

On the basis of the results of these and other experiments, it is believed that the specific substances more recently isolated and studied are substantially free from other similar materials and that the most active preparations are the specific blood group substances in a highly purified form. It seems doubtful, however, if any preparation will be found truly homogeneous if examined thoroughly enough by a combination of physical, chemical and immunological methods. Indeed, the results of a detailed serological examination of a series of highly purified group substances have shown that the specific materials appearing in the secretions of individuals who belong to the same group within the ABO and Le^a systems, nevertheless possess individual specific macromolecules which differ from each other in the pattern of their serological specificities. These differences must be a reflection of a different surface conformation which in turn arises from the nature and configuration of certain relatively small chemical structures on the outer, or serologically reactive, surface of the specific macromolecule.

Active materials obtained from secretions and purified by

any of the foregoing procedures show the same qualitative composition. All substances, irrespective of their blood group specificity, contain the same four sugars, L-fucose, D-galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine and at least eleven amino acids three of which, threonine, serine and proline, make up more than half the total amino acids. Aromatic and sulphur-containing amino acids are absent, or present in relatively small amounts. The composition and general properties of the group specific complexes indicate that they belong to a class of materials known as mucopolysaccharides,

Table I

TYPICAL ANALYTICAL FIGURES FOR PREPARATIONS OF THE HUMAN BLOOD GROUP SUBSTANCES

	N %	*Fucose* %	*Acetyl* %	*Hexosamine* %	*Reduction* %
A substance	5 4	19	9 0	29	54
H substance	5 3	18	8 6	28	50
Le^a substance	5 0	12	9 9	32	56
B substance	5 6	16	7 0	24	52
AB substance	5 6	17	—	26	54
"Precursor" substance	5 2	1 6	—	25	50

materials composed of carbohydrate units and amino acids firmly bound together and not present as a loose aggregate of macromolecular polysaccharide and a large protein moiety. Each specific mucopolysaccharide is believed to be built up of carbohydrate chains tightly integrated with peptides. Some typical quantitative analytical figures for the group substances are given in Table I.

Whether or not sialic acid is a component of the native blood group substance is less certain, as this residue is very readily split off by many mild procedures such as heating in aqueous or slightly acid solution. There is, however, no evidence that sialic acid plays any part as a serologically specific blood group structure, and in the most active and

highly purified preparations of the group specific mucopolysaccharides it is present to the extent of less than 0·3 per cent.

A more thorough examination of the procedure whereby the specific mucopolysaccharide could be recovered from secretions by treatment with hot saturated ammonium sulphate showed that if the method was applied to specific substances which had been obtained by the usual phenol extraction procedure, it separated many of the materials examined into two components, one insoluble in saturated ammonium sulphate at about 60°, and the other soluble under these conditions. When this was first observed the change suggested that a protein material, either a part of the original mucopolysaccharide or an unrecognized contaminant, was being liberated, denatured and thrown out of solution but the major part of the preparations appeared in the material precipitated, which was subsequently found to be readily soluble in water and was similar in composition to the original substance. Repeated treatment of each component recovered with hot saturated ammonium sulphate failed to convert the soluble material to a substance which was insoluble in saturated ammonium sulphate and *vice versa.* It seemed, therefore, that these two materials were not being changed, the one into the other, under the conditions used but were present in the original mucopolysaccharide preparation. The recovery and analysis of each substance (Table II) revealed that the material soluble in ammonium sulphate possessed a somewhat lower nitrogen content, a lower relative viscosity and a smaller capacity to inhibit the agglutination of erythrocytes by the appropriate group-specific agglutinin than did the original mucopolysaccharide. The substance insoluble in the hot saturated ammonium sulphate, on the other hand, had a higher nitrogen content, a higher relative viscosity and an enhanced specific serological activity than did the starting substance. In most other respects the two substances were shown to be closely similar, and the analytical figures in a typical experiment using a purified specimen of A substance as starting material

are given in Table II. A group A substance, and the two mucopolysaccharide materials separated from it, were examined by the quantitative precipitin technique (see Kabat, 1956) and it was found that the mucopolysaccharide component insoluble in ammonium sulphate precipitated more antibody nitrogen at the point of maximum precipitation than did the soluble component, and this latter material never precipitated at any concentration as much antibody nitrogen as did the mucopolysaccharide insoluble in hot ammonium sulphate. The original specific substance precipitated an amount of antibody nitrogen between that precipitated by the two components but ultimately at higher concentrations attained the maximum value reached by the mucopolysaccharide which was insoluble in hot saturated ammonium sulphate. The type of precipitin curve shown by the soluble material is usually considered to demonstrate that the substance under test which precipitates less antibody nitrogen is not contaminated with a non-specific component but is showing a different degree of reactivity, usually considered as cross-reactivity.

Table II

ANALYTICAL FIGURES FOR THE MUCOPOLYSACCHARIDE COMPONENTS SEPARATING FROM PURIFIED GROUP A SPECIFIC SUBSTANCE BY TREATMENT WITH SATURATED AMMONIUM SULPHATE AT 60–70°

	*Original mucopolysaccharide**	*Saturated ammonium sulphate*	
		Insoluble	*Soluble*
Nitrogen (%)	4 7	5·3	4·4
Fucose (%)	19	18	21
Amino sugar (%)†	29	29	30
Reduction (%)‡	52	52	56
Viscosity (Rel)	2 4	4 3	1·8
$[\alpha]_D$ (C, 1% in H_2O)	+2°	+10°	−18°
Serological activity (µg)§	0 012	0·006	0·40

* Laboratory Standard A material for serological titrations
† As glucosamine base after hydrolysis for 16 hr. at 100° with 0 5 N-HCl
‡ As glucose after hydrolysis for 16 hr at 100° with 0 5 N-HCl
§ Minimum amount giving inhibition of haemagglutination.

The finding that preparations of blood group substances specific for each of the serological characters associated with

the ABO and Le^a systems, can be separated into component mucopolysaccharides and that the same two materials can be obtained without the use of phenol and directly from the native ovarian cyst fluid indicates that these substances are present in the original secretion and are not artifacts of the isolation and purification procedures employed.

These results have helped us to understand many interesting, but hitherto unexplained, observations made with highly purified specific blood group substances. Repeated efforts to demonstrate impurity in the H substance obtained, for example, from cyst fluid No. 278, which contained only 3·9 per cent N, failed and yet this material possessed a much lower serological activity, measured by several reagents of different origin, than did the specific substance from cyst fluid No. 277 of similar specificity, but which contained 5·4 per cent nitrogen (Morgan, 1958). In the light of the behaviour of different specific mucopolysaccharide preparations on treatment with saturated ammonium sulphate at 60°, it was anticipated that substance 278 would be largely soluble in hot saturated ammonium sulphate whereas the more active material 277 would be insoluble. These predictions proved to be correct, and it was furthermore possible to show that material insoluble in ammonium sulphate at 60° was also largely insoluble in cold 90 per cent phenol, whereas the ammonium-sulphate soluble component was almost completely soluble in the phenol reagent; it was less active, and was only precipitated on the addition of ethanol.

The amounts of the two forms of specific mucopolysaccharide present in a preparation of the group substance isolated by the phenol method varies considerably in materials from different cyst fluids, and it is not known if the smaller mucopolysaccharide, that which is soluble in hot ammonium sulphate or more soluble in 90 per cent phenol, is derived from the larger and more active macromolecular complex by enzymic breakdown *in vivo*. It is possible that the proteases in the tissues bring about a hydrolysis of the larger molecular weight specific substance. However, at the moment, there is

no evidence for this suggestion, and it might well be that the relatively less active and smaller mucopolysaccharide is, in fact, secreted as such.

Degradation of the group specific substances with ficin and papain

It has been shown (Pusztai and Morgan, 1958) that the proteolytic enzymes, papain and ficin, bring about a modification in certain properties of the group specific mucopolysaccharides. The changes brought about involve reduction in viscosity, a decrease in the capacity of the materials to inhibit haemagglutination and a lowering of the nitrogen content. There is no evidence that any carbohydrate component is liberated by the enzymes. These changes have now been studied in detail. For example, a preparation of group A substance isolated from a pseudomucinous ovarian cyst fluid and believed to be substantially homogeneous, was incubated overnight at pH 4 in buffer with 1 per cent $^{w}/_{v}$ of three times crystallized papain activated with B.A.L. (2 : 3-dimercaptopropanol). The material was then dialysed, recovered and treated again with enzyme under the same conditions. The material finally recovered was fractionated from solution by the addition of ethanol. The analytical figures for each fraction and for the original untreated specific substance are given in Table III. The most striking change observed was that the original substance which precipitated sharply from 2 per cent aqueous solution between 45 and 50 per cent $^{v}/_{v}$ ethanol could now be separated on the addition of ethanol into several fractions, each more soluble under these conditions than the unchanged material. The largest fraction, which was seldom less than 50 per cent of the specific substance, precipitated between 50 and 55 per cent ethanol. Fractions were collected between 55 and 60 per cent, 60 and 75 per cent ethanol, and a few per cent of the original weight of material was recovered from the 75 per cent ethanol solution. These modified materials, although not grossly dissimilar in

chemical composition from the substance before treatment with papain, nevertheless all showed a much reduced viscosity and capacity to inhibit the agglutination of A erythrocytes by anti-A (α) agglutinin. Repeated treatment of each of these materials with papain did not further decrease their activity. The fall in serological activity is more evident when the substances are titrated for inhibition against human anti-A (α) serum, but most other anti-A reagents also indicate that a decrease in activity has occurred. The first fraction precipitated on the addition of ethanol, when titrated in inhibition tests

Table III

ANALYTICAL VALUES FOR THE PRODUCTS OF THE ACTION OF FICIN ON BLOOD GROUP A SUBSTANCE

	Original A substance	*Fractions precipitated from ficin-treated A substance*			
		Ethanol concentration (%)			
		50–55	55–60	60–75	*above* 75
Nitrogen (%)	5·3	4·6	4·8	4·8	5·2
Fucose (%)	19	21	21	20	16
Hexosamine (%)	28	29	28	26	22
Reducing sugar (%)	49	54	55	52	50
Viscosity (Rel.)	3·12	1·67	1·24	1·12	—
Haemagglutination inhibition activity μg	0·025	0·8	12	50	50

using normal human anti-A agglutinin, showed no more than a few per cent of the activity of the original substance. The other fractions precipitated possess less and less activity with increasing ethanol levels and the material recovered from the 75 per cent ethanol supernatant showed an activity of about 0·1 per cent of the original group substance. The behaviour of these materials with an immune rabbit anti-A (α) precipitating serum was studied according to the quantitative precipitin technique of Heidelberger and Macpherson (1943*a* and *b*), and Kabat and Bezer (1945). The amounts of antibody nitrogen precipitated by the addition of increasing quantities of each fraction to a rabbit immune anti-A serum were plotted against the amounts of material added. If the maximum amount of

antibody nitrogen precipitated by the original group substance is taken as 100 per cent, the materials, after treatment with papain, precipitate less antibody nitrogen at all concentrations examined. In this instance, the first fraction precipitated about 80 per cent and the last, the ethanol-soluble material, only about half the total antibody. Other preparations of A substance have yielded ethanol-soluble materials which precipitate no more than 10 per cent of the antibody precipitated by the original substance. Similar results have been obtained with substances treated with three times crystallized ficin. The antibody not precipitated by larger quantities of the degradation products of the specific substance after treatment with papain or ficin, can be readily precipitated on the addition of the original specific substance. The materials obtained from preparations of the specific substance by treatment with hot saturated ammonium sulphate solution, as described earlier, each behaved similarly on treatment with papain or ficin and showed a decrease in viscosity, in nitrogen and in serological activity, as measured by the inhibition of haemagglutination or by the capacity of the materials to precipitate antibody nitrogen. Material soluble in 75 per cent ethanol, which is poorly active serologically, is able to inhibit precipitation of the other, larger products of digestion, or of the original group-specific substance when these materials are mixed with a rabbit anti-A precipitating serum. Since the material soluble in 75 per cent ethanol presumably possesses intact oligosaccharide units, it is not surprising that it combines with and inhibits the precipitating action of the serum.

It is evident that the change induced in the specific mucopolysaccharides by ficin or papain is quite different from that brought about by the enzymes of microbial origin for, whereas these latter enzymes cause the mucopolysaccharides to liberate simple sugars and lose completely their specific serological properties, ficin and papain most probably act by bringing about a rupture of a limited number of peptide bonds, with or without the release of small amounts of amino acids or small

peptides, and with extensive but incomplete destruction of the specific serological character. These results suggest that the secondary or gross macromolecular structure plays an important part in the quantitative serological behaviour of the specific mucopolysaccharide, perhaps by bringing about the orientation of the serologically active carbohydrate units to the requirements of the receptor sites on the antibody molecules.

The serologically active structures of specific mucopolysaccharides

In the belief that only a small part of the large group-specific mucopolysaccharide molecule is responsible for the serological specificity shown by the whole macromolecule, attempts have been made to determine which part of the complex structure carries the specific determinant groupings.

The most obvious approach is to break down the specific substance with mild acid or alkali and isolate and identify any units which retain some of the original serological specificity. The difficulty of controlling the extent of the breakdown of the specific complexes, which are extremely sensitive to acid or alkaline hydrolysis, and the absence of means whereby units containing more than three or four different sugars linked together in different ways can be completely separated from each other with any degree of certainty, has restricted the results so far obtained by this approach. Furthermore, quantities of several grams of the specific substance are required to yield milligram amounts of each of the oligosaccharide hydrolysis products.

The small molecular weight products formed by the gentle hydrolysis of group A substance with dilute mineral acid have been examined after their separation on charcoal: celite and cellulose columns (Côté and Morgan, 1956 and unpublished data). By this method six nitrogen-containing disaccharides were recovered and identified by direct comparison with disaccharides of known structure or from their own chemical and chromatographic behaviour. These compounds are listed

in Table IV, and it will be appreciated from the fact that it is possible to isolate this number of different disaccharide units, which does not necessarily account for all the possible combinations of sugar units present, how very complicated are the carbohydrate structures in group A mucopolysaccharide. The limited but important immunochemical information given by these disaccharides will be discussed later.

Recently, methods have been introduced which give similar information but do not require the destruction of the group substances and moreover require only mg. amounts of the materials. One approach was based on an early observation (see Landsteiner, 1947) which showed that substances of small

Table IV

N-CONTAINING DISACCHARIDES ISOLATED FROM GROUP A SUBSTANCE

(1) *O*-α-D-galactosyl-(1 → 3)-*N*-acetyl-D-galactosamine
(2) *O*-β-D-galactosyl-(1 → 3)-*N*-acetyl-D-glucosamine
(3) *O*-β-D-galactosyl-(1 → 4)-*N*-acetyl-D-glucosamine
(4) *O*-α-*N*-acetyl-D-galactosaminoyl-(1 → 3)-D-galactose
(5) *O*-β-*N*-acetyl-D-glucosaminoyl-(1 → 3)-D-galactose
(6) *O*-α-L-fucosyl-(1 → 6)-*N*-acetyl-D-glucosamine

molecular weight with identical or closely similar structures to those possessed by the immunologically active group in a complex antigen, were able to combine with the antibody and thus inhibit the reaction between the antigen and its antibody. The closeness of the relationship of the structure of the simple test-substance to the determinant structures of the antigen are reflected in the firmness with which the simple substance is bound by the antibody. An estimate of this specific relationship is obtained by measuring the amount of the simple substance required to give inhibition under a set of standardized test conditions and, using this technique, it was found that L-fucose, alone of all the sugars present in H substance and of many other sugars tested, inhibited the agglutination of O cells by an eel anti-H serum and suggested that L-fucose played a special part in H specificity (Watkins and Morgan,

1952). The examination of the inhibiting capacity of methyl α- and β-L-fucopyranosides and the α- and β-fucofuranosides showed that the α-L-fucopyranosyl structure gave the strongest inhibition. Similar inhibition experiments were also made using an anti-O cell agglutinin of plant origin extracted from *Lotus tetragonolobus* seeds, and again the results indicated that an α-L-fucopyranosyl structure played a specific rôle in H specificity (Morgan and Watkins, 1953). The inhibition of the agglutination of A cells by plant seed anti-A reagents obtained from *Vicia cracca* likewise gave an early clue to the part played by *N*-acetylgalactosamine in A specificity.

Human or rabbit anti-H or anti-A agglutinins are usually not inhibited, or inhibited very weakly, in their action on O or A cells, respectively, by these simple sugars and it seems that the receptor sites of these antibodies are directed against a more extensive pattern of chemical structure on the specifically reacting surface of the group substance and are thus more exacting in their structural requirements than are the eel and plant seed agglutinins. Some support for this suggestion comes from the observation that although, for example, *N*-acetylgalactosamine inhibits the agglutination of A cells by an extract of *Vicia cracca* seeds it does not inhibit the agglutination of A cells by human anti-A serum, whereas the disaccharide, α-*N*-acetylgalactosaminoyl-(1→3)-galactose isolated from human A substance, does bring about inhibition of both anti-A reagents (Côté and Morgan, 1956). Kabat and Leskowitz (1955) using a more precise precipitation technique and a human anti-A precipitating serum were also able to demonstrate the importance of *N*-acetylgalactosamine in A specificity and extended the studies to the B anti-B system and showed that an α-D-galactoside structure would inhibit the precipitation of B substance by a human anti-B precipitin.

Independent evidence to establish the nature of the simplest structure within each group-specific mucopolysaccharide which plays a dominant part in the group-specific determinant structure was obtained from the results of enzyme inhibition experiments in which the serological inactivation of a specific

substance by the enzyme was prevented by the presence of a simple sugar. Enzymes obtained from *Trichomonas foetus* (Watkins, 1953, 1956, 1959) or from *Clostridium welchii* (Stack and Morgan, 1949; Buchanan, Crumpton and Morgan, 1957) and which rapidly destroyed the serological specificity of the blood group substances were used in these experiments. The inactivation of A substance was prevented by *N*-acetylgalactosamine and its methylglycoside and, to some extent, by D-galactose, but not by any of the other sugars included in the test. The inactivation of B substance by the *T. foetus* enzyme, on the other hand, was only slightly inhibited by *N*-acetylgalactosamine, but the same concentration of D-galactose, the methyl-α- and β-galactopyranosides and certain disaccharides containing galactose inhibited the action of the enzyme strongly. The serological inactivation of H substance by both enzymes could be inhibited by L-fucose and D-galactosamine. It is of interest to find that the methyl-fucopyranosides and -furanosides fail to inhibit the inactivation of the H substance whereas the galactopyranosides inhibit the enzyme which destroys B substance. A partially purified H active enzyme from *T. foetus* is, however, without action on the methylfucosides although the presence of a fucosidase in the enzyme preparation seems certain as fucose is rapidly and extensively liberated from H substance. These results suggest that the sugars which bring about the inhibition observed are those which play an important part in determining the specificity of the appropriate blood group substance.

It is of interest to note that the potentialities of the enzyme and antibody inhibition techniques are different in that the former can give no more than an indication of the terminal sugar involved in specificity, whereas the latter can, if a series of suitable test substances are available, be used to elucidate both the nature and the sequence of the sugars which constitute the major part of the complete determinant structure.

An interesting example of the way in which these techniques have been used to obtain evidence for the type of chemical

structure that is responsible for Le^a specificity can now be given. Le^a substance, the specific substance secreted by the so-called non-secretors, is rapidly inactivated by an enzyme preparation obtained from *T. foetus* and the inactivation can be prevented by L-fucose, but not by the other sugars present in Le^a substance. At first sight, this observation suggests a relationship between H and Le^a specificity because the H substance and H antibody reaction is also inhibited by L-fucose. Serologically, however, these two substances are quite distinct and it seems that other structural differences, such as the mode of linkage of the L-fucose or the nature of the sugars adjacent to it, exist between H and Le^a substances In the light of the evidence already provided by the enzyme and antibody inhibition experiments, it can be assumed that L-fucose plays a definite rôle in Le^a specificity, but it is found that L-fucose has no detectable capacity to inhibit the agglutination of Le (a+) red cells by a human anti-Le^a serum. On the assumption that the antibody required a more extensive pattern of chemical structure, possibly a larger portion of the essential oligosaccharide chain, than a single non-reducing L-fucosyl end-group, a number of oligosaccharides which contained fucose were examined for their power to inhibit the agglutinating action of the serum on Le (a+) cells. Many of the oligosaccharides tested were isolated by Prof. Kuhn and his colleagues (see Kuhn, 1957) and their exact structure determined. The constitutions and trivial names of these compounds, which were kindly made available to us by Prof. Kuhn, are given in Fig. 1.

The inhibition tests included Lacto-*N*-fucopentait II, which is the alcohol of Lacto-*N*-fucopentaose II, and three fucose-containing disaccharides, α-L-fucosyl-(1→2)-, -(1→3)- and -(1→4)-L-fucose (Côte, 1959). It is believed that the fucose in all the substances tested is present in the pyranose form. Many other simple sugars and disaccharides at concentrations of up to 20 mg./ml. were included in the tests but none gave inhibition. The results of the tests are given in Table V, from which it will be seen that Lacto-*N*-fucopentaose II and its

alcohol, in which the terminal glucose is reduced to sorbitol, alone possessed a high capacity to inhibit human anti-Le[a]

1 Lacto-N-tetraose

O-β-D-galactosyl-(1→3)-*O*-β-D-*N*-acetyl-glucosaminoyl-(1→3)-*O*-β-D-galactosyl-(1→4)-D-glucose

2 Lacto-N-fucopentaose I

O-β-D-galactosyl-(1→3)-*O*-β-D-*N*-acetylglucosaminoyl-(1→3)-*O*-β-D-galactosyl-(1→4)-D-glucose
(galactosyl substituted (1→2) by α-L-fucosyl)

3. Lacto-N-fucopentaose II

O-β-D-galactosyl-(1→3)-*O*-β-D-*N*-acetylglucosaminoyl-(1→3)-*O*-β-D-galactosyl-(1→4)-D-glucose
(glucosaminoyl substituted (1→4) by α-L-fucosyl)

4. Lacto-N-difucohexaose

O-β-D-galactosyl-(1→3)-*O*-β-D-*N*-acetylglucosaminoyl-(1→3)-*O*-α-D-galactosyl-(1→4)-D-glucose
(galactosyl substituted (1→2) by α-L-fucosyl; glucosaminoyl substituted (1→4) by α-L-fucosyl)

5. Fucosido-lactose

O-β-D-galactosyl-(1→4)-D-glucose
(galactosyl substituted (1→2) by α-L-fucosyl)

6. Fucosido-galactose

O-α-L-fucosyl-(1→2)-D-galactose

7. Lacto-difucotetraose

O-β-D-galactosyl-(1→4)-D-glucose
(galactosyl substituted (1→2) by α-L-fucosyl; glucose substituted (1→3) by α-L-fucosyl)

FIG. 1.

serum. Lacto-*N*-tetraose fails to bring about inhibition, a result which supports the belief that L-fucose plays a definite part in Le[a] specificity. The strongly active Lacto-*N*-fuco-

pentaose II differs from the tetraose only in containing an *α-L-fucosyl radical joined by a branching (1→4) linkage to the* penultimate sugar residue, *N*-acetylglucosamine. Lacto-*N*-difucohexaose, which contains an additional *O*-α-L-fucosyl unit attached by a (1→2) linkage to the non-reducing *O*-β-D-galactosyl end-group is much less active than is Lacto-*N*-fucopentaose II so that the proximity of a second fucose molecule appears to impair the specific relationship of the active Lacto-*N*-fucopentaose II oligosaccharide to the Le^a antibody. The full inhibiting capacity shown by the Lacto-*N*-fucopentait II indicates that the reducing glucose end-unit plays no significant part in the Le^a specificity shown by Lacto-*N*-fucopentaose II. The inhibition results also allow one to conclude that when an *O*-α-L-fucosyl unit is joined by a (1→2) linkage to a galactose residue, as in Lacto-*N*-fucopentaose I, fucosidolactose or *O*-α-L-fucosyl-(1→2)-galactose, it fails to induce any capacity to neutralize the Le^a antibody. The results suggest

Table V

THE AMOUNTS OF Le^a SUBSTANCE AND SUGARS GIVING INHIBITION WITH HUMAN ANTI-Le^a SERUM

Substance	*Minimum amount of substance giving inhibition* (μg /0 1 ml.)
Human Le^a substance	0·04
L-Fucose	>1,000
D-Fucose	> 1,000
α-Fucosyl-(1 → 2)-fucose	> 1,000
α-Fucosyl-(1 → 2)-galactose	> 1,000
Fucosidolactose	> 1,000
Lacto-*N*-tetraose	> 1,000
Lactodifucotetraose	> 1,000
Lacto-N-fucopentaose I	> 1,000
Lacto-*N*-fucopentaose II	1
Lacto-*N*-fucopentait II	1
Lacto-*N*-difucohexaose	60

strongly that the serologically active structure in Lacto-*N*-fucopentaose II resides in the specific spatial pattern provided by the structure given in Fig. 2, and that the high potency as an inhibitor shown by Lacto-*N*-fucopentaose II makes it probable that this branched structure is very close structurally to the naturally occurring carbohydrate configuration in Le^a substance (Watkins and Morgan, 1957). Although confirmation of this suggestion must await the

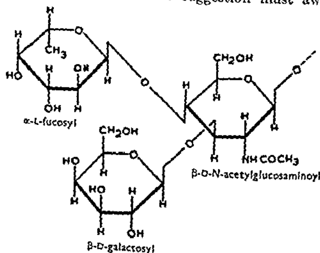


FIG. 2.

results of a detailed study of the fine structure of the oligosaccharide chain in the Le^a substance which is responsible for the specific character, it is of interest to note that a serologically reactive trisaccharide which contains fucose, galactose and *N*-acetylglucosamine, has been isolated by Dr. J. Thomas at the Lister Institute, from the products of mild (pH 8·5) alkali hydrolysis of Le^a substance.

In view of the low capacity to inhibit shown by the simple sugars or disaccharides which are active in the A, B or H inhibition tests compared with the activity of the branched structure in the Le^a tests, it might be anticipated that branched carbohydrate chains in which two non-reducing

end-groups attached to the penultimate sugar residue, or some other sugar not too far removed from the non-reducing end group in an oligosaccharide, will be found to comprise the reactive structure in other serologically specific substances.

If the conclusions reached on the structures believed to be responsible for group specificity on the basis of the results of serological and enzyme inhibition tests are correct, then further support for them might be forthcoming from the results of similar inhibition tests in which the six *N*-disaccharides isolated from the acid hydrolysis products of group A substance, and described earlier in this communication (Table IV), are used as test substances. It will be seen that one of them (No. 4) has a non-reducing end-unit which is believed to constitute the dominant serological structure in A-specific substance. Of the six disaccharides and of many others tested for inhibition, only substance No. 4, *O*-α-*N*-acetyl-D-galactosaminoyl-(1→3)-D-galactose, inhibited anti-A serum, a result which completely supports the conclusions drawn from the antibody and enzyme inhibition experiments already described.

The A, B, H and Le^a substances, after short, mild hydrolysis at pH 2–3, liberate fucose and react strongly with Type XIV pneumococcus antibody, and it has been concluded that the removal of fucose uncovers structures which are reactive with the pneumococcal antibody (Kabat *et al.*, 1948). The pronounced cross-reactivity suggests that, as galactose and *N*-acetylglucosamine are components of all the group substances and of the pneumococcus Type XIV polysaccharide, the overlapping reactivity arises from the presence of a common galactose-*N*-acetylglucosamine structure. Support for this idea was obtained when it was found (Watkins and Morgan, 1956) that the disaccharide (Table IV, No. 3) *O*-β-D-galactosyl-(1→4)-*N*-acetyl-D-glucosamine, inhibited the precipitation of slightly degraded blood group substance with Type XIV pneumococcus antiserum and that this disaccharide unit is present in pneumococcus Type XIV polysaccharide (Barker *et al.*, 1958).

Conclusions

The chemical nature of the substances responsible for the specific immunological characters associated with human blood group specificity has been established in general terms during the last two decades. The substances are mucopolysaccharides, macromolecular complexes built up of carbohydrate chains and amino-acid units integrated into a stable molecular species by primary chemical bonds. In the light of recent serological findings, however, in which multiple group specificity is believed to be associated with a single mucopolysaccharide molecule, it is desirable that several methods should be available for the isolation and purification of these important biological units and that the criteria for judging their homogeneity should be extended. Recent progress in this direction has been discussed and the influence of macromolecular structure on the serological activity of the specific substances has been considered.

Although much work remains to be done on the fine structure of the group specific mucopolysaccharides, some progress can be reported which associates a definite chemical structure with a particular serological specificity in each specific substance within the ABO and Le^a blood group systems. The weight of evidence obtained from different laboratories and by different techniques allows one to conclude that:

(a) ABH and Le^a specificities are associated with carbohydrate structures on the surface of mucopolysaccharide macromolecules.

(b) A specificity arises from non-reducing *O*-α-*N*-acetylgalactosaminoyl-(1→3)-galactosyl end-groups.

(c) B specificity is associated with *O*-α-D-galactosyl end-units.

(d) H specificity is primarily due to *O*-α-L-fucosyl end-residues.

(e) Le[a] specificity most probably arises from a branched trisaccharide unit containing non-reducing *O*-β-D-galactosyl and *O*-α-L-fucosyl end-groups joined (1→3) and (1→4), respectively, to *N*-acetylglucosamine.

(f) Pneumococcus Type XIV activity is associated with a *O*-β-galactosyl-(1→4)-*N*-acetylglucosamine structure.

(g) Amino acids are not directly involved in the specific determinant structures in the group specific substances.

There is, therefore, much evidence for the belief that certain carbohydrate units within the specific substances are primarily responsible for specificity, but it has not yet been possible to determine the exact nature and sequence of the sugars which constitute the complete determinant structure for any one blood group specificity. Evidence is accumulating that not only the nature and sequence, but also orientation and integration within the macromolecule of the essential carbohydrate units play an important part in determining the ultimate degree of activity of the specific substance. In the light of the results of certain enzyme studies, it seems probable that this spatial integration is brought about by the amino-acid residues or peptide chains contained in the specific substance.

The group substances possess a characteristic immunological specificity which is gene-controlled and which sharply differentiates one type of mucopolysaccharide from another. These materials are, therefore, of great value as "chromosome" markers and can be used to study certain problems in human genetics at a biochemical level, such as gene interaction, and to suggest possible pathways for the biosynthesis of the specific substances. These and other aspects are considered by Dr. Winifred Watkins in the following communication.

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[Discussion of this paper was postponed until after the paper by Dr. Winifred M. Watkins.—EDS.]

SOME GENETICAL ASPECTS OF THE BIOSYNTHESIS OF HUMAN BLOOD GROUP SUBSTANCES

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AMONGST the human gene products which have received detailed biochemical study, the blood group substances present two unusual features. The first is the fact that the structures determined by the blood group genes confer a high degree of immunological specificity on the molecules. In the absence of these specific properties it is extremely doubtful whether it would be possible to distinguish one blood group substance from another; the A, B, H and Le^a substances are all mucopolysaccharides which are very similar in their general chemical and physical properties, and each is composed of L-fucose, D-galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and the same eleven amino acids (see Kabat, 1956; Morgan, 1959*a*). The immunological specificity, however, provides an infallible criterion for distinguishing the different blood group gene products, and a study of the structures which determine specificity therefore gives an insight into the chemical nature of the units synthesized under the influence of the genes.

Evidence which has accumulated in recent years suggests that these structures are associated with the carbohydrate portions of the mucopolysaccharide molecules and that the peptide moieties probably play no part in specificity other than determining the correct orientation of the specific carbohydrate groupings (see Morgan, 1959*b*). The second aspect, therefore, in which the human blood group substances differ from the other human gene products which have received detailed biochemical study, such as the haemoglobins

(see Itano, 1957; Ingram, 1959) and the haptoglobins (Smithies, 1955; Smithies and Walker, 1956; Bearn and Franklin, 1958), is that the genetically determined structures appear to be carbohydrate rather than protein in nature.

According to current theories (see Crick, 1958), the genetical material consists of deoxyribonucleic acid (DNA) which is arranged in such a way that it provides "information" for the precise determination of the amino-acid sequence of proteins; proteins are therefore considered to be the only direct products of gene action. If these ideas are correct the formation of the blood group specific carbohydrate structures must be at least one stage removed from the blood group genes and the function of these genes must be assumed to be the formation of specific protein enzymes which subsequently effect, or control, the carbohydrate synthesis. This being so, it would seem improbable on theoretical grounds that one gene could be responsible for the complete synthesis of the group specific mucopolysaccharide molecules which not only have four different sugars to be built into the carbohydrate chains but also contain amino acids firmly bound in the molecule by primary valency bonds.

The concept that the blood group genes act late in the synthesis of the specific mucopolysaccharides and do not control the formation of the complete molecule had been arrived at earlier from general considerations of the nature and properties of these materials. Thus, from the fact that approximately equal amounts of mucopolysaccharides were present in the saliva and gastric juice in all individuals irrespective of whether they were secretors or non-secretors, and that these materials could not be differentiated by any known chemical test, Wiener and Wexler (1952) suggested that the blood group genes act by bringing about some minor modification in the molecules of a mucopolysaccharide substrate which all human beings share in common. Gibbons and Morgan (1954) also suggested that the *ABO* and Lewis genes should be considered as genes which control a stage, or series of stages, in the building up of certain substances by the body,

modifying them so that the substances finally produced, possess the configuration which is associated with A, B, AB or Lewis specificity according to the genotype of the individual. Apart from the similarity in qualitative chemical composition and physical properties of the blood group substances derived from individuals belonging to different ABO and Lewis groups, there is evidence that they share certain structural chemical groups since the human A, B, H or Le^a substances, either in the native state or after mild degradation with acid or enzymes, each have the capacity to cross-react with Type XIV anti-pneumococcus horse serum (see Kabat, 1956). This cross-reactivity is attributed, at least in part, to the presence in each of the substances of β-linked 1 : 4-galactosyl-*N*-acetylglucosamine units (Watkins and Morgan, 1956; Schiffman, Howe and Kabat, 1958) and this finding is compatible with the view that the blood group genes modify a common mucopolysaccharide substrate. The most convincing evidence, however, that the individual blood group genes do not control the complete synthesis of the final mucopolysaccharide molecules has been the demonstration that more than one specificity can occur on a single molecular entity (Morgan and Watkins, 1956; Watkins and Morgan 1956–57; Watkins and Morgan, 1959; Brown, Glynn and Holborow, 1959).

The fact that the secretions of individuals belonging to group AB who are secretors inhibit the agglutination of both A and B cells by their respective homologous antisera has been known since the earliest work on the secretor phenomenon (Lehrs, 1930; Putkonen, 1930; Schiff, 1931). Until recently, however, it was not known whether the secretions in these heterozygous individuals contain a mixture of molecules, some of which are A-specific and others B-specific, or whether they contain molecules which carry both A- and B-specific groupings. As mentioned above, the A and B substances are closely similar in their chemical and physical properties and it is, therefore, not feasible to attempt to separate them by the usual chemical fractionation methods or by electrophoretic or ultracentrifugal procedures. The method used to obtain

evidence for the existence of mucopolysaccharide molecules with single or multiple specificities, therefore, was that of serological precipitation tests with selected specific antisera. When the specific material in the secretions of a group AB individual was precipitated with an anti-A reagent—which was completely free from B precipitins—it was found that the

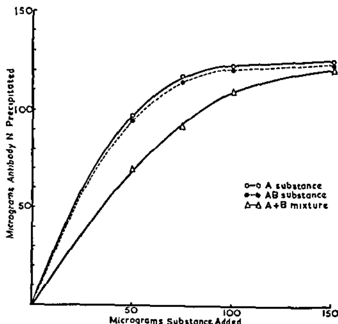


FIG 1. Precipitin curves for A substance, AB substance and a mixture of A and B substances with a rabbit anti-A serum.

redissolved precipitate had both A and B activity whereas when the same procedure was applied to an artificial mixture of A and B substances, only the A activity was carried down in the precipitate (Morgan and Watkins, 1956). Subsequent experiments have shown that the A and B activities remain together in the same ratio when the precipitates obtained with AB substances are redissolved and reprecipitated three times. Similar results are obtained when the first precipita-

tion is made with an anti-A reagent and the second with an anti-B reagent, or *vice versa*. Estimation, by the method of Heidelberger and Macpherson (1943*a* and *b*) of the antibody nitrogen precipitated from a rabbit anti-A serum by given amounts of purified specimens of A and AB cyst substances gave curves which were almost identical (Fig. 1). The same

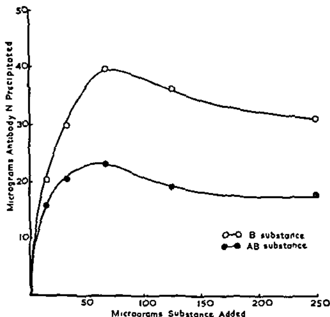


FIG 2. Precipitin curves for B substance and AB substance with a rabbit anti-B serum

amount of each substance was required to give maximum precipitation of antibody and the maximum reached was practically the same for the two substances, whereas a mixture composed of equal amounts of A and B substances gave a clearly distinguishable curve. The AB substance precipitated only 50–60 per cent of the antibody nitrogen precipitated from a rabbit anti-B serum by B substance (Fig. 2) but again the amount of each substance required to give maximum

precipitation was the same (Morgan and Watkins, 1959*a*). The behaviour of the AB substance in the quantitative precipitation test is in agreement with the restricted B serological activity observed earlier for AB preparations in agglutination inhibition experiments (Gibbons and Morgan, 1952) and would support the suggestion made by Gibbons, Morgan and Gibbons (1955) that if the A and B active structures were situated together on the same macromolecule they could inhibit each other's sphere of action owing to steric interference.

The interpretation that we have put on the foregoing results is that, in the secretions and tissue fluids of AB secretor individuals, the majority of the mucopolysaccharide molecules have both A- and B-specific groupings and that these result from the interaction of the *A* and *B* genes (Morgan and Watkins, 1956). An alternative explanation is that the A and B substances are formed separately under the influence of their respective genes and that the products aggregate or polymerize after their formation Allison (1959) has recently put forward a theory which involves secondary association forces to explain the hybrid molecules which are found in the sera of individuals heterozygous at the haptoglobin locus. It is important to distinguish between the two possible interpretations of the results found for AB substances because if the second explanation were correct the problem, although of practical interest, would be of little genetical significance. Although the possibility cannot be absolutely excluded that association between preformed A and B molecules takes place within the cell, it has not been possible to demonstrate such an association between mixtures of A and B substances *in vitro*, nor has it been possible to obtain any evidence that AB substances can be dissociated into molecules of A and B substances. For example, when samples of A and B saliva, which have received no form of treatment, are mixed immediately after collection, the two specific activities can be completely separated by repeated precipitation with either anti-A or anti-B precipitating reagents. This is true whether the saliva

mixtures are added immediately to the precipitating serum or if they are kept at either 0° or 37°, for 24 hours before precipitation. Under these conditions, therefore, association of the molecules does not take place. Attempts to bring about dissociation of the AB molecules by application of ultrasonic vibrations has also been unsuccessful. Treatment of the AB substance with ultrasonics under such severe conditions that a fall in A and B serological activity was observed, failed to bring about any demonstrable dissociation of the A and B activities. We therefore favour the interpretation that in the heterozygote the A and B genes collaborate to produce a different molecular species from that formed when either gene is present in the homozygous state.

Further examination of the blood group specific materials in secretions revealed that the occurrence of more than one specificity on the same molecule does not arise solely from the interaction of allelic genes. The H-specific character is not believed to arise from the action of a gene allelic with A or B (see Watkins and Morgan, 1955*a*) but, nevertheless, in secretions of individuals belonging to the sub-group A_2, where fairly potent H activity invariably accompanies the A activity, there are molecules which carry both A and H serological specificity as well as some which have H specificity only (Watkins and Morgan, 1956–57). The proportion of free H appears to vary from one individual to another. In addition, the Lewis Le^a character, which is inherited independently of the ABO system (see Race and Sanger, 1958), can also be shown to occur on the same molecule as the A, B or H characters (Morgan and Watkins, 1959*b*; Brown, Glynn and Holborow, 1959). In the secretions of group A secretors showing both A and Le^a activity, for example, there are molecules which possess both A and and Le^a specificity as well as molecules which have only Le^a activity. The failure of rabbit anti-Le^a sera to precipitate with Le^a when it occurs together with A, B or H substances in the saliva of secretors, also observed by Brown, Glynn and Holborow (1959), has prevented the examination of ALe^a secretor saliva for the

presence of molecules which show only A activity. Similarly, in the absence of powerful precipitating anti-H reagents it is not possible to determine whether free A molecules are present in the secretions of A_2 individuals. The important point, however, is that molecules with dual specificity have been shown to exist even when the specificities involved are believed to result from the activities of independent gene systems. It seems highly probable that, in fact, mucopolysaccharide molecules with multiple, i.e. more than two, specificities can occur. In the secretions of a group A_2B individual who also secreted Le^a substance, for example, the existence of molecules each of which carried A, B, H and Le^a specificity, could be postulated; indeed preliminary experiments have supported this assumption.

The implications of these findings are of considerable practical importance from the point of view of a biochemical approach to the nature of the secreted mucopolysaccharide materials and also must be considered in the application of quantitative precipitation tests to these substances. The question which I wish to consider in more detail here, however, is the possible biosynthetic pathway by which molecules with multiple specificities could be formed. On the basis of the biochemical, serological and genetical data available on the water-soluble blood group substances, Prof. Morgan and I have suggested two schemes for the later stages of the biosynthesis of these specific mucopolysaccharides (Watkins and Morgan, 1959). It is improbable, for reasons to be given later that the first scheme, outlined in Fig. 3, is tenable but the necessity for introducing, on genetical grounds, the modification which resulted in scheme II (Fig. 4) will be clearer if scheme I is discussed first.

The generally accepted theory of the inheritance of the ABO groups is that advanced by Bernstein (1924) according to which the blood group of an individual depends on the presence of any two of three allelic genes *A*, *B* and *O*; the ability to secrete A, B or H substances in saliva was also shown by Schiff and Sasaki (1932) to be inherited as a Mendelian

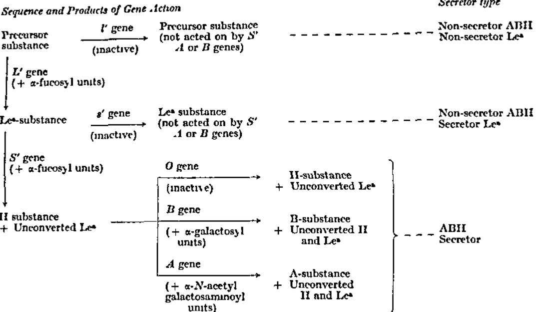


FIG. 3. Suggested genetical pathways for the biosynthesis of the water-soluble mucopolysaccharides (Watkins and Morgan, 1959)

dominant character and the genes concerned were designated *S* and *s*. A relationship between the secretor phenomenon and the Lewis Le^a character of the red cell was established when Grubb (1948) observed that all persons whose red cells were Le(a+) were non-secretors of A, B or H substances, and that

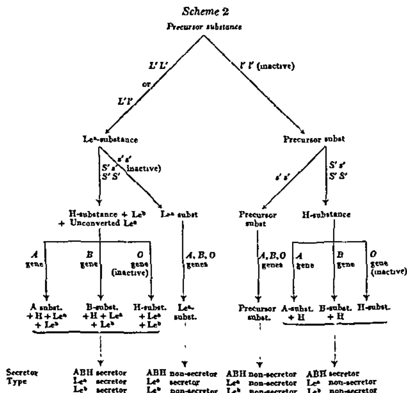


FIG. 4. Alternative pathways for the biosynthesis of the water-soluble mucopolysaccharides (Watkins and Morgan, 1959).

Le^a substance was present in the saliva of these Le(a+) individuals. Later, Ceppellini (1955*a*) put forward a theory to explain the relationship between the Lewis groups and ABH secretion which was based on two pairs of alleles, *S* and *s*, which controlled the appearance, or non-appearance, of the A, B and H substances, and *L* and *l* which determined

the presence or absence of Le^a substance in secretions. We propose that the appearance of ABH and Le^a substances in the secretions depends on the operation of three independent gene systems, L' and l', S' and s', and A, B and O. The symbols S and s used by Schiff for secretion have been retained because we think that the appearance of the water-soluble A, B and H substances in secretions is dependent on the possession by an individual of the S' gene, but the superscript has been added because the genes are considered to be transforming genes determining a step in the biosynthesis of the mucopolysaccharides; the function normally attributed to the gene S is that of allowing the conversion of the ABH substances to a water-soluble form. Similarly, the symbols L and l used by Ceppellini for the presence or absence of Le^a substance have been retained but again the superscript has been added because a somewhat different function for these genes is envisaged and the phenotypic effects resulting from the combinations of the genes proposed in scheme I (Fig. 3) do not completely correspond with those resulting from Ceppellini's theory.

In the schemes, the genes L', S', A and B are transforming genes which control certain stages in the conversion of a precursor substance to the specific products which appear in the secretions. The genes l', s' and O play no part in the conversion of the mucopolysaccharide materials, i.e. as far as their part in the scheme is concerned they may be considered as inactive genes or amorphs. The scheme given in Fig. 3 also requires that the transformations brought about by the genes can take place only in the sequence shown and that no further change in the material can occur in the absence of one of the conversion steps. The precursor substance is considered as a macromolecular mucopolysaccharide with all the peptide chains completely synthesized and with most of the carbohydrate chains already integrated in the molecules. In individuals homozygous for the inactive gene l' the precursor material can undergo no further change since, in the absence of the conversion step effected by the L' gene, the subsequent gene transformations brought about by the S', A and B genes

do not occur; individuals homozygous for gene *l'*, therefore, correspond to the group who are non-secretors of A, B, H, Le^a or Le^b and any mucopolysaccharide material in their secretions should correspond to the precursor substance. Examination of a material from such an individual showed that the product was very similar in its general chemical composition to the active blood group substances except for a very low fucose value (1·6 per cent) and the only serological reactivity of this "inactive" material so far demonstrated is the capacity to cross-react with anti-Type XIV pneumococcus serum. It is, therefore, suggested that the precursor material has prominent β-galactosyl-*N*-acetylglucosaminoyl structures, most probably glycosidically linked by 1 : 4 bonds. The subsequent transformation steps mask or overshadow these groupings, but they are revealed again in A, B, H or Le^a substances after mild degradation and, in consequence, such degradative processes give rise to the development of Type XIV reactivity.

The action of the gene *L'* is to convert the precursor substance into Le^a substance and, as fucose has been shown to play an important part in Le^a specificity (Watkins and Morgan, 1957), it is envisaged that this step involves the addition of non-reducing α-fucosyl units to the precursor mucopolysaccharide. This is assumed to hold for the gene combinations *L'L'* and *L'l'*. The very low fucose content of the "inactive" mucopolysaccharide and the substantially higher fucose content of typical Le^a preparations (12–14 per cent) would be in agreement with the suggested function of the *L'* gene. The gene *S'* in single or double dose controls the conversion of Le^a substance to H substance and, as fucose also plays a rôle in H specificity, this transformation step is believed to involve the addition of further α-fucosyl units, probably through different linkages and to different sugars from the changes effected by the *L'* gene. It seems probable that this conversion is not usually complete so that the product will retain some residual Le^a specificity in addition to its newly acquired H specificity. The results of the precipitation experiments

suggest that this could arise either through some Le^a molecules remaining unchanged and/or through residual Le^a specific structures remaining unaltered on molecules which have acquired H reactive structures.

The mode of inheritance of the Le^b character and its relationship to the ABO and Le^a groups is still not completely understood (see Race and Sanger, 1958). Ceppellini (1955*a*) considers that Le^b specificity is the product of interaction of the genes *S* and *L* and, since Le^b seems to be closely related to the H character in its serological properties, it might be that the *S'* gene converts Le^a to both H and Le^b substances by the addition of fucose units to different structures or side-chains in the Le^a macromolecule. Preliminary observations on the inhibition by simple carbohydrates of the agglutination of Le(b+) cells by human anti-Le^b serum has suggested that fucose plays a part in Le^b serological activity (Watkins and Morgan, 1957).

Individuals with an *L'* gene who are homozygous for the inactive genes *s's'* produce Le^a substance which can undergo no further change because the *A* and *B* genes, even when present, require H as a substrate and cannot act directly on Le^a substance. These individuals, therefore, correspond to the group of ABH non-secretors who, nevertheless, contain large quantities of Le^a substance in their secretions.

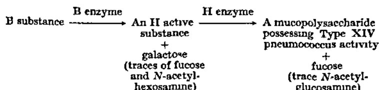
We consider the *A* gene, in either single or double dose, to control the conversion of the preformed H substance into A substance by the addition of non-reducing α-*N*-acetylgalactosaminoyl units since there is definite experimental evidence that this unit is involved in A specificity (Morgan and Watkins, 1953; Kabat and Leskowitz, 1955; Watkins and Morgan 1955*b*; Côté and Morgan, 1956). Any residual Le^a specificity remaining with the H substance will appear in the secretions, together with A substance as well as unchanged H substance. The A_2 gene is believed to bring about the same conversion as the A_1 gene, but to do so less effectively so that more H-specific structures remain untransformed and are, therefore, available for reactivity with anti-H sera.

B specificity has been shown to be associated with the presence of α-galactosyl units in the mucopolysaccharide molecule (Kabat and Leskowitz, 1955; Watkins and Morgan, 1955*b*) The *B* gene, therefore, in either single or double dose is thought to direct the addition of non-reducing α-galactosyl units to H substance to give a B substance which, according to the extent of conversion, contains residual H and Le^a specificities. In heterozygous AB individuals, the *A* and *B* genes both act on the H substrate to convert it to an AB substance and, therefore, individuals of this genotype would be expected to contain very little residual H specificity, as is indeed found experimentally. The *O* gene is regarded as inactive and, as a consequence in individuals homozygous for this gene, large quantities of unchanged H substance are found in the secretions, together with free or combined residual Le^a and Le^b specificities A combination of the genes *L′* and *S′* together with an *A*, *B* or *O* gene, therefore, gives rise to the group of ABH secretors who also secrete both Le^a and Le^b substances.

Three of the four main groups into which secretors can be divided on the basis of the secretion of Lewis and A, B, H substances (Ceppellini, 1955*a*) are accounted for by scheme I, i.e. (1) secretors of A, B or H, Le^a and Le^b substances, (2) secretors of Le^a substance, non-secretors of Le^b and A, B or H substances and (3) non-secretors of ABH, Le^a or Le^b substances. The fourth secretor group, which constitutes some 10 per cent of the population, i.e. secretors of ABH, non-secretors of Le^a (and possibly of Le^b) could also be accommodated in this scheme if it were assumed that different *S′* genes, possibly alleles related to each other in the same way as A_1 and A_2 genes are related, can bring about varying degrees of conversion of Le^a to H. In the presence of very effective genes, no residual Le^a structures would remain and, therefore, the final product found in the secretions after the action of the *A*, *B* or *O* genes would be devoid of Le^a activity.

The synthetic pathway outlined in Fig. 3 would account for the striking similarity in qualitative chemical composition of the blood group materials, irrespective of the group of the

individual from whom they were derived, for the nature of the structures responsible in each substance for its characteristic specificity and for the fact that two or more different specific structures can occur on the same molecule. The observation that A and B substances can be converted *in vitro* by enzymes into mucopolysaccharides which are devoid of their original specificity, but which develop H specificity (Iseki and Masaki, 1953; Iseki and Ikeda, 1956; Watkins, 1956) provides experimental evidence to support the suggestion that the H active mucopolysaccharide is the substrate on which the *A* and *B* genes, or perhaps more correctly specific enzymes produced under the influence of these genes, act. The changes in serological specificity observed during the decomposition of group B substance by *Trichomonas foetus* enzymes (Watkins, 1956) are as follows:



The development of Type XIV cross reactivity by A, B and H substances after mild acid hydrolysis or degradation with enzymes is consistent with the structure suggested for the precursor substance. In addition, Le^a substances which are considered to be nearer to the precursor substance than the more extensively converted A, B or H substances normally show a greater capacity to cross-react with Type XIV serum in the native state.

The first scheme, therefore, fits well with many of the biochemical facts which are known about the water-soluble human blood group mucopolysaccharides and is not at variance with the serological observations on secretion. However, with the sequence of gene action suggested in this scheme, a mating of an ABH non-secretor, Le^a non-secretor, i.e. an individual of the genotype *l'l'S'S'*, with an ABH non-secretor,

secretor of Le^a i.e. an individual of the genotype *L'L's's'*, can give rise to an ABH secretor (genotype *L'l'S's'*). This combination would presumably occur fairly infrequently but exceptions to the rule that non-secretors of ABH cannot give rise to secretors do not seem to have been recorded (see Race and Sanger, 1958) The modification to the scheme, shown in Fig. 4, has been introduced to overcome this objection. The concept that the genes can act only in a given sequence and that in the absence of one conversion step the succeeding steps cannot take place, has been modified to allow the gene *S'* to control the conversion of either Le^a or the unchanged precursor substance to H substance. It can be further postulated that when the *S'* gene acts on Le^a substance, Le^b specific structures are produced, in addition to H, whereas H structures alone arise as the result of the action of *S'* on the precursor substance. The four types of secretor groups can, therefore, be accounted for by this scheme without postulating the existence of *S'* genes of differing effectiveness. The possible combinations of the two allelic pairs, *L'* and *l'*, and *S'* and *s'*, for this second scheme are the same as those postulated for the genes *L*, *l*, *S* and *s* in Ceppellini's theory. The second scheme, still largely based on biochemical observations on the blood group mucopolysaccharides, will thus be seen to be in very close agreement with the genetical ideas elaborated on the basis of serological results on family material (Ceppellini, 1955*a* and *b*; Ceppellini and Siniscalco, 1955).

It will be apparent that neither of the schemes given here can account for the appearance of the blood group characters on the red cell surface since the A, B and H antigens are, with only extremely rare exceptions, always present on the red cell when an individual carries the *A*, *B* or *O* genes. Some different mechanism must operate, however, to bring about the divergence in phenotypic expression between the blood group character of the red cell and of secretions, and a sequence of steps which is applicable to the synthesis of the secreted materials need not necessarily be the same as that which leads to the formation of the red cell antigens. Isolation of group

specific materials from red cells has proved difficult and the chemical nature of the blood group substances as they occur on the intact cell is still not established (see Kabat, 1956). While it seems fairly certain that the actual group specific structures on the red cell antigens will be chemically identical with those on the water-soluble substances it is not known whether these specific structures are part of the same type of mucopolysaccharide molecules as are found in secretions. It is therefore conceivable that at the site of formation of the red cell antigens the synthetic steps mediated by the blood group genes could take place using different substrates from those occurring in secretions. In order to account for the appearance of the antigens on the red cells, however, and particularly to explain the occurrence of H antigen on the red cells of group O non-secretors i.e. individuals who, according to the schemes, lack the S' gene necessary to convert Le^a or the precursor substance to H, it may be necessary to suggest a different rôle for S' and s' from that given in the schemes. Thus, instead of a transforming gene which brings about a step in the synthesis of the mucopolysaccharides, S' may be considered as an inactive allele and s' as a gene, functioning only during the course of synthesis of the secreted blood group substances, which, when present in double dose, suppresses the transformation step which converts Le^a or the precursor substance to H substance. The suggested addition of α-fucosyl units to Le^a or the precursor substance could be brought about by an independent gene *H* which with very rare exceptions is possessed by all individuals. If the genes $s's'$ are unable to suppress the action of the *H* gene at the site of synthesis of the red cell antigens the H substance would invariably be formed when an individual possessed this gene, and the subsequent transformations to A and B could then proceed when the appropriate gene was present. Those very rare individuals who lack A, B and H reactivity both in their secretions and on their red cells i.e. the "Bombay" phenotype (see Race and Sanger, 1958) could then be homozygous for a rare allele of *H*, namely *h*, which fails to bring about the

conversion step carried out under the influence of the *H* gene. The secretor and red cell types resulting from the action on the precursor substance of the various possible combinations of the genes *L′*, *l′*, *H*, *h*, *S′*, *s′* and *ABO* are given in Table I. It is of interest to note that if this theory is correct there should be two distinct groups of "Bombay" phenotypes, namely, those who lack ABH antigens on their red cells but secrete Le^a substance in their saliva and a more rare group who also lack

Table I

SUBSTANCES IN THE SECRETIONS AND ON THE RED CELLS RESULTING FROM THE ACTION ON THE PRECURSOR SUBSTANCE OF COMBINATIONS OF THE GENES *L′*, *l′*, *H*, *h*, *S′*, *s′*, *A*, *B* and *O*.

Gene combinations	*Substances in secretions*			*ABH antigens on red cells*	
	ABH	Le^a	Le^b		
L′, H, S′, ABO	+	+	+	+	
L′, H, s′s′, ABO	−	+	−	+	
L′, hh, S′ or *s′s′*, *ABO*	−	+	−	−	"Bombay" phenotype
l′, hh, S′ or *s′s′*, *ABO*	−	−	−	−	"Bombay" phenotype
l′, H, s′s′, ABO	−	−	−	+	
l′, H, S′, ABO	+	−	−	+	

ABH antigens on their red cells but who fail to secrete Le^a in their saliva.

It should perhaps be emphasized that the pathways suggested in the schemes concern only the later stages of the biosynthesis of the blood group mucopolysaccharides, namely the possible steps by which the blood group specific structures become incorporated in the molecule. Very little is known about the biogenesis of mucopolysaccharides, and it is not possible to speculate on the origin of the precursor substance. Moreover, although the precursor mucopolysaccharide substrates are thought to have certain constant structural features, such as the groupings which give rise to Type XIV specificity, we do not mean to imply that there is necessarily only one precursor material on which the *L′* or *S′* (or *H*) genes can act. Indeed, Gibbons and Morgan (1954), on the basis of the isolation of two substances from a single group B cyst,

both of which showed B character and yet possessed different physical, chemical and serological properties, suggested that the A and B genes modify a number of different mucopolysaccharide materials which are part of the normal mucinous secretions of the body. The findings of Pusztai and Morgan, reported in this symposium (Morgan, 1959*b*), that purified blood group substances of all groups can be divided by ammonium sulphate precipitation into two fractions differing in their serological, chemical and physical properties, could also be explained on the basis of there being more than one precursor substance.

For the sake of simplicity, the steps in the conversion of the precursor substrate controlled by the genes have been described in the schemes as the addition of those non-reducing glycosyl units which have been found to play a dominant rôle in the specificity of the serological character in question. Although such a function would not be inconsistent with the present state of our knowledge regarding polysaccharide synthesis *in vitro* by means of specific enzymes (see Whelan 1956–57) the changes controlled by the genes may well be more complex. Allen and Kabat (1957) found that after mild acid hydrolysis of A, B and H substances each developed a new and independent specificity; these results indicate that the group specific substances differ from each other by more than those terminal non-reducing sugars which are important for specificity. In addition, our present knowledge of the relationship between chemical structure and immunological specificity in the blood group field suggests that, although the terminal sugars play a dominant rôle in a specificity, a sequence of different sugars, possibly in the form of branched structures, will be found to constitute the complete serologically determinant units. Further developments in the elucidation of the relationship between genes and carbohydrates are needed before it is possible to decide how such structures could be built up under the influence of genes; if the "one gene-one enzyme" concept of Beadle (1945) is correct, however, it would appear that several genes would be required

for the formation of one complete determinant unit. The genes *L′*, *S′*, (*H*) *A* and *B* could then be regarded not as single genetic units but each as a series of closely linked genes which produced a series of enzymes determining the nature, sequence and linkages of the sugars in the serologically specific structures, and the genes *l′*, *s′* (*h*) and *O* could be mutants which failed to produce one or more of the specific enzymes.

Whatever the mechanism which effects their synthesis the blood group specific structures provide examples of genetically determined characters of carbohydrate nature in which the sugar sequence and configuration is presumably exactly determined and precisely limited. The problems encountered in the chemical study of these materials, the type of interactions which can occur between genes at the same or different loci, and the variations in the molecule resulting from a gene mutation are likely to be considerably different from those found for the gene products which are protein in nature. Thus, although possibly less closely related to the genes which determine their structure than are the genetically determined proteins, the blood group mucopolysaccharides have an important and independent contribution to make to the study of human biochemical genetics.

Summary

Serological precipitation experiments have demonstrated that the blood group specific mucopolysaccharides in secretions and tissue fluids can have more than one blood group specificity associated with the same macromolecule. The specificities can arise from the action of allelic genes, such as A and B or from the action of genes at independent loci, such as A and Le^a. In the light of these results, and other biochemical and serological data, possible genetical pathways for the biosynthesis of the blood group specific mucopolysaccharides are discussed.

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DISCUSSION

Grubb: I wonder if the gene *l′* is an amorph or if it is responsible for the appearance of a precursor substance?

Watkins: The gene *l′* cannot be responsible for the appearance of the precursor substance because, according to the scheme, individuals who do not possess this gene, i.e. individuals who are homozygous for the allele *L′*, nevertheless have precursor substance which is converted to Le^a substance by the action of the *L′* gene. It is therefore suggested that the precursor mucopolysaccharide material is formed at an earlier stage in the biosynthetic pathway and that the *l′* gene is an amorph which brings about no further change in this substance.

Lederberg: How well-ordered is the polysaccharide? Could it be understood as a product of a mélange of enzymes putting on the various side chains wherever the configurational specificities admit, or do you have to postulate a definite serial assembly?

Morgan: It is not possible to answer your question completely, because we do not yet know the detailed structure of the specific substances. We know that all the group mucopolysaccharides, irrespective of serological specificity, contain the same four sugars in roughly the same amounts, so that there is no selection from this point of view. Each substance is completely distinct serologically, and therefore there is some control that always goes on in a group A person, for example, which determines that certain of the carbohydrate chains shall end with a non-reducing α-*N*-acetylgalactosaminoyl unit attached most probably by a 1 : 3 linkage to galactose.

Watkins: We would also believe that there is a precisely determined sequence responsible for specificity.

Lederberg: Let me put it a little differently. Is there a more definite structure than could be inferred from knowing all the component disaccharide segments?

Watkins: Yes.

Lederberg: Could you enlarge on this?

Morgan: The general body of evidence concerning the immunological specificity of carbohydrate structures indicates that whereas the non-reducing end unit plays a dominant part in the serologically specific character of the material, the second and subsequent sugar units and their linkages also contribute to the specificity; the contribution of each sugar residue having a smaller and smaller influence the further they are from the serologically dominant non-reducing end unit. Of the six N-containing disaccharides isolated from group A substance, only one has any A specificity.

Watkins: And that has only a very small fraction of the total activity of the whole specific unit.

Lederberg: I am not questioning the specificity of the antibody which may well be specific for larger sequences. Does the antigen contain only such ordered segments, or are these occasional features in a more random conjunction?

Morgan: As we do not know how long the carbohydrate chains are or whether there is more than one type of chain in each specific substance, it is not possible to say if specifically ordered oligosaccharide sequences exist throughout the substance.

Lederberg: Do you know whether the blood group substance consists only of such repeating units as long, say, as four residues?

Morgan: It is not possible to say if there is a repeating tetrasaccharide unit.

Kalmus: The word precursor denotes a definite sequence of reactions in a biochemical scheme. There seems to be some doubt about the special arrangements of some of those sequences. Could not different sequences explain why different antigens occur in the same person at the surface of the red cells and in the secretions? Also could not similar syntheses be effected by simultaneous reactions?

Morgan: The ordered sequence of gene action suggested in the scheme has been put forward to account for the biochemical and serological facts. It has been found possible, for example, to degrade A and B substances enzymically and to obtain newly developed H activity whereas it has not been possible to detect A or B activity on degradation of H substance. It is therefore suggested that H is the substrate on which the *A* and *B* genes act. The order of the other steps has been suggested for similar reasons. It does not seem probable that a different order of gene action could explain why different

antigens occur in the same person at the surface of the red cells and in the secretions.

Brenner: It is possible that some degree of retention of a longer sequence may occur for purely steric reasons. This argument depends on a paper by Corey (1959) in which this question was asked: why, if glycogen is made by a random polymerization process, does it attain a relatively homogeneous size? The answer seems to be reasonable, i.e. the units are put in until a certain packing is reached when the reactive groups are no longer accessible, or relatively less accessible. This would for purely steric reasons fix the size of the molecule. One other point is: would it not be possible, since you have all these apparently blocked precursors, to search for the enzymes in tissue extracts that will convert one precursor to another? Then one would have direct evidence for what the enzymic activity is, rather than using the degradation technique. This is of course the classical chemical method, or at least the first stage of it. One should look for the biosynthetic enzyme, because this goes to the heart of the matter.

Morgan: We have mainly worked so far with hydrolytic rather than synthetic procedures and have attempted to determine structure by this means. The problems in using enzymes as a tool for this purpose are considerable because the enzyme preparations available which decompose the group substances possess many carbohydrase activities and bring about extensive breakdown of the substances after the changes which lead to a loss of serological specificity have occurred, and in consequence the results are difficult to interpret. Progress in this direction will depend largely on the success with which carbohydrase preparations possessing a single hydrolytic activity can be obtained.

Brenner: In your scheme you had a blocked precursor, i e. if the gene was absent a precursor had accumulated. If the gene was present there was an enzyme which attacked a certain group. It seems to me that you can get both the mutant cells and the wild-type cells; you have the precursor which you can isolate, and serological tests for the product. You can now compare the two cells and try to find an activity in one which is absent in the other. This seems to be the more direct approach.

Watkins: We have the synthetic approach in mind. Indeed, experiments along the lines you suggest have been tried but as yet without success. Another method is to attempt to synthesize serologically active structures using known transglycosidases of bacterial or protozoal origin. However, until the structure of the blood group substances is known or, at least until the sequence of sugars in the serologically specific structures has been determined, it is not possible

to know which enzymes to use. Also if the addition of sugars takes place by a transglycosylation mechanism, it is necessary to have the appropriate donor substrates and this introduces a further difficulty because glycosides and oligosaccharides containing fucose or hexosamines are not readily available.

Ingram: I wonder if Prof. Morgan and Dr. Watkins can give us a model of these substances, their picture of what the molecule is like.

Morgan: The carbohydrate chains are joined to peptides by primary chemical bonds and are thus forced to assume a spatial configuration which can be considered as a secondary structure. The macromolecule on treatment with ficin or papain loses this secondary structure to some extent, presumably through the hydrolysis of a few peptide bonds, with consequent partial loss in capacity of the specific substance to react with antibody The carbohydrate chains are, so far as we know, not altered or split off by this treatment.

Ingram: How many of these specific sites do you think there are in one molecule?

Morgan: The number of serologically specific non-reducing sugar units in the molecule of mucopolysaccharide is not known.

Kalckar: Kuhn reported recently on his joint work with Prof. Morgan [Kuhn, R. (1959). *Geigy Coll. I, Modern Problems in Pediatrics*, p. 25. Basel: Karger] It is interesting that a branched pentaose containing fucose is immunologically active as Lewis's hapten. The enzyme which catalyses the formation of the cross-reacting pentaose might be present in milk. A study of such types of enzymes may give more direct information at the genetical level. If there were "secretors" of Lewis antigen it would be useful for a study of enzymes of the branched pentaose.

PHYSIOLOGICAL GENETICS OF HUMAN BLOOD FACTORS*

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BLOOD factors may be defined as inherited discrete characteristics of the red cell structure which are present in certain individuals of a species and lacking in others. At present, they can be recognized only by means of serological reactions and thus they are mainly regarded as antigens; in effect, they can induce the formation of specific antibodies when erythrocytes carrying the factor are injected into individuals of the same species (isoimmunization) or of another species (heteroimmunization) which do not possess this particular structure. But it must be realized that the term antigen, or better the operational result of one or more serological reactions, only qualifies one characteristic of the substance, i.e. the ability of eliciting and reacting with specific antibodies, but *per se* does not give any information about the function, chemical nature or molecular size of the substance in question. For instance, it is not known whether the genetically related Rh specificities C and D correspond to one or two different molecules. Now we know that the chemical configuration which is responsible for the serological specificity,

* *Terminology:*

ABO blood group system. Phenotypes: A, B, AB, O. Antigens: A, B, H. Genes: I^A, I^B, i^O (abbreviated to *A*, *B*, *O*).

ABH secretion system. Phenotypes: SH = presence in saliva of H (and possibly A and/or B) specificity, nh = absence of such specificity. Genes: *Se*, *se*.

Lewis system. Phenotypes of red cells = Le(a b). Phenotypes of saliva: Les = presence of Le^a or Le^a+Le^b specificity; nl = absence of such specificity. Genes: *Le*, *le*.

Suppressor system: The phenotype corresponding to homozygosity for the very rare allele *x* is designated as "Bombay" or Oh. All usual ABO blood groups are assumed to be homozygous or heterozygous for the common *X* allele. (The *X*–*x* genes of Levine and co-workers, 1955, correspond to the *H*–*h* genes of Watkins and Morgan, 1955).

i.e. the antibody-combining site or area of the "antigen" to which the antibody is complementary, may be, and probably often is, only a small portion of the antigenic molecule. Here, the studies of Kabat (1957) are particularly informative, having shown that the antibody-combining site of the huge dextran molecule is limited to a short sequence of from 4 to 6 1 : 6-α-linked glucopyranose units. It can thus be easily visualized that one macromolecule, i.e. one chemical entity, can carry more than one serological specificity. Moreover, the variety of antibodies that an antigen can elicit upon injection into a suitable recipient is strongly limited (or "canalized") not only by its own structure, but by the antigenic composition of the recipient. That is particularly true in the case of iso-immunization where donor and recipient share many serological specificities: for instance, the injection of CC blood into a cc recipient produces a specific anti-C serum, while injections of cc blood into a CC recipient produce anti-c, but injection of homozygous CC or cc blood into an individual who lacks both C and c, for instance a D−−/D−− (so-called homozygous deletion), also produces an anti-Cc antibody (Hackel, 1957), which can react with both the "allelic" substances, showing in them the presence of a common specificity, usually undetected with the "pure" antisera.

It is worth noting that in some respects this is quite fortunate, because in formal genetical analysis we are mainly interested in emphasizing the differences among genetical alternatives, and the specification of all common parts would be pleonastic.

When, on the other hand, a phenogenetic interpretation must be attempted on the basis of serology alone, the limitations of the method become clear: for instance, how will it be possible to define the *Rh* cistron, the gene as a physiological unit, when the Rh phenotype is only partially and conventionally known through some of its serological specificities?

Nonetheless, already in the early development of immunogenetic studies it was postulated that the antigens of erythrocytes represent an immediate product of the controlling genes

(Irwin and Cole, 1936; Haldane, 1938), and more recently Horowitz (1956) mentioned them as good examples of the template theory of gene action; the main basis for these assumptions being the apparent absence of interaction of genes at different loci in controlling the specificity of cellular antigens and the regular inheritance of minor variants of antigens, such as C^w or E^u, which are suggestive of the genetical determination of the finest details of the antigen structure.

As regards the lack of interaction, it must be realized that the phenomenon may be only apparent and of evolutionary origin; for instance, a final phenotype may be the result of a number of steps, each one genetically controlled.

$$A_1A_1 \rightarrow B_1B_1 \rightarrow \begin{matrix} C_1C_1 \\ C_1C_2 \\ C_2C_2 \end{matrix} \rightarrow \text{"observed" phenotypes } \mathrm{C}\ (11 \text{ or } 12 \text{ or } 22)$$

But at only one of the many loci more than one allele exist in the wild population, while at all other loci one "best" allele is fixed: from the Mendelian point of view C segregates as a straightforward monofactorial trait, but interaction does exist.

According to the nature of human material, where lethal mutants are not easily brought to parenthood, maintained in stock and crossed at will, the possibility that such situations will escape detection is certainly common.

In effect, a typical case of epistasis and two-factor inheritance has been observed and properly interpreted, involving the ABO antigens, although it is limited to their expression in some mucoid secretions, typically saliva. Some years later a close relationship was observed between ABO secretions and another system of blood and secretion antigens, the Lewis system; finally, another independent locus, *Xx*, influencing the expression of the *ABO* genes both in red cells and in saliva has been detected. These four independent loci interact as a synergistic physiological system giving rise to the dynamic picture of a metabolic sequence ending in the produc-

tion of a complex macromolecule with more than one serological specificity. Its interpretation has been greatly facilitated by the opportunity, unique in immunogenetics, that some data—which are very valuable although at the moment incomplete—are available on the chemical structure of the phenotype involved.

The ABO Blood Groups

The basic facts about the genetics of the ABO blood groups need not be discussed, but a few comments are necessary concerning the real nature of the O blood group and of the *O* gene. According to the standard technique of the ABO typing, the group O is recognized by negative reactions with both anti-A and anti-B sera. Because these two reagents are unable to distinguish *AA*, *BB* homozygous from *AO*, *BO* heterozygous cells, the *O* gene is correctly regarded as completely recessive in respect to *A* and *B* (Bernstein, 1924). A number of reagents—natural or immune—of human, animal or vegetable origin agglutinate preferentially O (and A_2) cells, but they also react to various degrees (although generally more weakly) with all bloods of groups A and B. As a result of the hypothesis of a one to one relationship between the gene and the antigen, a great deal of effort has been spent in an attempt to select among these heterogeneous reagents the ones which would detect the specific product of the O gene, i.e. the ones which would distinguish between erythrocytes of genotype *AA* (BB) and *AO* (BO).

It must be concluded that such sera have not been found; because of the heterogeneous distribution of the serological specificity of the antisera which react preferentially with the group O cells, they are now better known as anti-H.

The writer does not consider of much use the distinction between anti-O and anti-H according to their ability of being inhibited by different soluble blood group substances, and regards as more important the serological heterogeneity of anti-H sera which probably detect different although related specificities of the ABO antigens. (For a complete discussion

and detailed bibliography see the excellent review of Watkins and Morgan, 1955.)

The various degrees of reactivity of different A(B) cells with anti-H sera seem to be inversely proportional to the amount of A specificity (the less A specificity the more H) and are largely, although not completely, independent from the AA or AO phenotype of the individual (Grubb, 1949). Although extensive studies on the correlation of H reactivity among relatives of the same non-O blood group have not been done following the pioneer work of Hirszfeld and Amzel (1940), the impression has been gained that the H reactivity mainly depends on the greater or lesser efficiency of the *A(B)* alleles for imparting a new serological specificity to a pre-existing substrate; if this is the case, while the *A* and *B* alleles can be regarded as morphic genes, the *O* allele is the amorph and the $i^O i^O$ genotype corresponds to a metabolic block which ends up with the accumulation of the substrate. Because the H specificity is characteristically present in highest amounts in the blocked $i^O i^O$ genotype, H may be identified with the substrate upon which the *A* and *B* genes act. As will be pointed out later, a number of genetical and biochemical observations support this hypothesis which, at the moment, seems to be the most economical. Consequently, there is no need for the existence of a specific O antigen.

Distribution of ABH antigens in tissues and secretions: the ABH secretion system

The ABH antigens are present in very small amounts on the surface of the red cell as alcohol-soluble, water-insoluble substances. By means of the new and elegant technique of "mixed agglutination", the existence of AB receptors has been established beyond any doubt on platelets (Coombs and Bedford, 1955), on epidermal cells (Coombs, Bedford and Pott, 1956) and on desquamated cells of the amniotic fluid (Frieslcben, 1956), of all individuals. It is probable, but not yet proved, that the majority of cell types possess the ABH

antigens in this alcohol-soluble form. In some individuals, but not in others, water-soluble substances with ABH serological specificity are found in high concentration in some bodily secretions and aqueous tissue extracts, chiefly in saliva, gastric duodenal and seminal juice, sublinguary and submaxillary glands (the parotid gland is poorer), gastric and duodenal mucosa and, in general, where muciparous cells are abundant.

The serological activity of soluble blood group substances is detected by means of an inhibition test, i.e. the ability of the substance to inhibit the agglutination of an indicator system represented by appropriate red cells and antiserum (A anti-A, B anti-B, O anti-H). The serological specificity in secretions, if present, corresponds (with a few exceptions) to the blood group of the subject; in addition, secretors of all blood groups besides group O can inhibit the anti-H sera; the ubiquitous presence of H specificity is still more marked in secretions than in erythrocytes.

In contrast, other individuals (about 20 per cent in Caucasians) do not show in their bodily secretions (and aqueous tissue extracts) any appreciable amount of A, B or H specificity; these are called non-secretors. It must be emphasized that there is no readily detectable chemical difference between the mucoid secretions of secretors (SH) and non-secretors (nh): the difference consists in whether some bodily components, the fucomucoids, are endowed with ABH specificity or not.

It has long been known that secretor (ABH) or non-secretor status is inherited as a simple Mendelian trait, by means of two pairs of alleles *Se* and *se*; *se* is completely recessive and thus the non-secretor phenotype corresponds to the *sese* genotype, while the secretor phenotype corresponds to *SeSe* or *Sese*. It was also shown that the *Se-se* locus is completely independent from the *ABO* locus (Ceppellini and Siniscalco, 1955).

We are faced with a first problem. The *ABO* genes have at least two functions: the induction of ABH specificity in the lipopolysaccharides bound to the stromal protein of the red cells, and the induction of similar specificity in the fucomucoids

of secretions. Is that a true example of pleiotropism? Probably not, because the primary action of the gene is likely to be the same and consists in both cases of the attachment to a main molecule of the same short side-chains from which the serological specificity derives. Little is known about the nature of the ABO-secretor co-operation, but the possibility should be considered that the *Se* gene differs substantially from all other known genes which determine antigenic specificity. No specific products of either *Se* or *se* are known (see below); in the writer's opinion the dominant *Se* gene does not add any new information of its own to the blood group substances, but only activates also in the muciparous cells the genes responsible for the making up of the ABH structures. That recalls some suppressor genes (*Su*) in *Neurospora* which, according to a recent interpretation (Suskind and Kurek, 1959), do not seem to contribute to the specific information of the enzyme, but modify the internal environment of the cell and thus increase the efficiency of an otherwise defective system produced by a mutant *Td* gene From the physiological point of view it is known that the production of blood group antigens in mucoid cells is many times greater than in erythrocytes.

Whatever may be the nature of this interaction, up to now the *Se* gene was thought to co-operate directly with the genes of the *ABO* locus. In the light of the most recent findings which suggest that the *ABO* genes act upon an H substrate, it is more likely that *Se* acts at the level of the *X–x* locus (see below). In fact, if the *sese* genotype corresponded to a post-H block, non-secretors of group A and or B would secrete no A or B antigens, but would still be able to secrete H specificity. That is why the new phenotypic symbols SH and nh have been proposed here.

The Lewis System

1. Reaction on red cells with anti-Le^a and anti-Le^b sera

An antiserum, now known as anti-Le^a, discovered by Mourant in 1946, allows a first classification between Le(a+)

and Le(a−) individuals with a frequency in the Caucasians of 20 and 80 per cent, respectively. The frequency of the two classes is the same among all ABO groups. In the majority of cases the Le(a+) trait seems to be inherited as a recessive; that is already peculiar because it is the only instance in Man of a *serologically positive* trait, which is not detected in the heterozygous (O anti-H reactions excluded for the reasons previously given). In fact the appearance of two Le(a−)nh children from an Le(a−)nh × Le(a−)nh mating has been reported (Ceppellini and Siniscalco, 1955). Another such family has been described more recently (Ceppellini, Dunn and Innella, 1959).

Shortly afterwards a second serum, now known as anti-Le^b, was detected by Andresen (1948), which reacts with about 70 per cent of group O and A_2 cells, but with a frequency much lower in other blood groups, chiefly group A_1. Different samples of anti-Le^b show a different frequency of reactivity with A_1; the same serum, on storage, may lose its initial ability of agglutinating A_1(b+) cells.

The reactions of anti-Le^b sera appear to be *almost* antithetical to the reactions of anti-Le^a in the sense that among *adults* of group O and A_2 all Le(a+) cells are Le(b−), hence Le(a+b−); and about 70 out of the 80 Le(a−) are Le(b+), hence Le(a−b+) Ten per cent of the total are negative with both sera, hence Le(a−b−). A third kind of serum, anti-X, or better anti-Le^x of Andresen and Jordal (1949), reacts with both Le(a+b−) and Le(a−b+) cells; it probably corresponds to the cross-reacting portion of the antibody population, often present in anti-Le^a sera.

The three Lewis phenotypes were initially (and still are by a number of authors) regarded as determined by a series of 3 alleles in the following order of dominance: Le^b, Le^a, Le^c. While the frequencies in the general population would be more or less in agreement with such a hypothesis, the family data are not, because Le(b+) offspring are obtained also from Le(b−) × Le(b−) matings.

2. Lewis and ABH secretions

In 1948, Grubb made the brilliant observation that people of the red cell phenotype Le(a+b−) are always non-secretors of ABH; (Le(a−b+) are always secretors; Le (a−b−), disregarding the epistatic effect of A_1 and/or B on red cell Le^b, are SH or nh in a ratio which is the same as in the general population.

Moreover, Grubb (1951) observed that (i) the mucoid secretions of Le(a+b−) have the Le^a specificity; (ii) also the secretions of Le (a−b+) individuals, who are SH, inhibit to some degree certain anti-Le^a sera; in the present writer's experience, the degree of inhibition depends mainly upon the reagent used and is independent from the alleged Le^bLe^b or Le^aLe^b genotype; (iii) all Le (a−b−) individuals, both SH and nh, do not show in their secretions any Le^a specificity.

Table I

INDEPENDENCE OF LEWIS SECRETIONS FROM ABH SECRETIONS IN SALIVA

	Les	*nl*	χ^2	*nh per cent*	*se*	*nl per cent*	*le*
Charleston[1]							
SH	138	40	0·07	22	0·50	25	0·52
nh	44	14					
Ferrara[2]							
SH	374	50	0·04	18	0·42	12	0·35
nh	83	11					
Sweden[3]							
SH	715	85	3·07	20	0·44	10	0·31
nh	187	13					

[1] Ceppellini, Dunn and Innella (1959)
[2] Ceppellini (1955*a* and *b*) [3] Grubb (1951)

Hence, if the positivity for a Lewis antigen, Le^a or Le^b, and the Lewis negativity are plotted in a 2 × 2 table against the ABH secretor or non-secretor status, a complete independence of the two traits is observed. That has been confirmed in three independent sets of data (Table I).

In an attempt to explain these observations, Grubb proposed in 1951 a very far-sighted scheme which at the time seemed too complicated to be accepted; it needed a number of subsidiary epistatic effects to fit all the facts, and it lacked the support of family investigations.

The writer was attracted by the problem of a possible interaction between more independent loci and decided to examine a large number of families (Ceppellini, 1955*a* and *b*; Ceppellini and Siniscalco, 1955). The first Italian investigation permitted, in agreement with Grubb, the following conclusions:

(1) The positivity for one of the Lewis factors, Le^a *or* Le^b, in the red cells or the equivalent ability to show some degree of Le^a specificity in saliva, is inherited as a simple Mendelian trait, dominant over Lewis negativity; hence, two genes, *Le* and *le*, are postulated. Le (a−b−), SH or nh, corresponds to the recessive *lele* genotype.

(2) The *Le-le* locus is not linked with the *Se-se* locus. No evidence for linkage with ABO or other blood groups was found.

3. The nature of the Le^b antigen

Using an anti-Le^b serum which, in respect to red cell reactivity, corresponded with Andresen's original one [positivity with 70 per cent of group O and A_2, negativity with all adults Le(a+), depressed or abolished reactivity with group A_1] Grubb (1951) observed that *all* secretors of ABH were able to inhibit such a serum; thus, this serum behaves as an anti-Le^b against red cells but as an anti-H in inhibition tests. Grubb concluded that the anti-Le^b serum reacts in saliva against a specific product of the *Se* gene, thus, Le^b, as detected with this kind of antiserum, is always present in the saliva of ABH secretors. Grubb did not discuss the discrepancy between red cells (Le b−) and saliva (Le b+) type in the class of Le(a−b−) SH.

The present writer was impressed by the fact that Le^b in the red cells is always present when saliva shows both Lewis and

H specificity, which are respectively indices of an *Le* and an *Se* gene. It is absent if only *Le* genes without *Se* genes are present (Le a+b− nh) and it is also absent when only *Se* genes without *Le* genes are present (Le a−b− SH). Therefore, it was tentatively supposed that, at least in red cells, the Le^b specificity arises from the co-operation of the two dominant *Le* and *Se* genes (Ceppellini, 1955*a* and *b*).

Table II

EXAMPLES OF H AND LEWIS SPECIFICITIES IN SALIVA OF DIFFERENT INDIVIDUALS. NOTE THE DISCREPANCY BETWEEN THE TWO TYPES OF ANTI-LE^b SERA

Red cell phenotype	*Indicator system*	*Saliva dilution* 2^0	1^1	2^2	2^4	2^5	2^6	2^7	2^8	2^9	2^{10}	*Saliva phenotypes*
Group O Le(a−b+)	O-Ulex	−	−	−	−	−	−	−	−	+	+	SH Les
	anti-Le^a	−	−	−	−	−	+	+	+	+	+	
	anti-Le^bH	−	−	−	−	−	−	−	+	+	+	
	anti-Le^bL	−	−	−	−	−	−	−	+	+	+	
Group O Le(a+b−)	O-Ulex	+	+	+	+	+	+	+	+	+	+	nh Les
	anti-Le^a	−	−	−	−	−	−	−	−	−	+	
	anti-Le^bH	+	+	+	+	+	+	+	+	+	+	
	anti-Le^bL	−	+	+	+	+	+	+	+	+	+	
Group O Le(a−b−)	O-Ulex	−	−	−	−	−	−	−	−	−	+	SH nl
	anti-Le^a	+	+	+	+	+	+	+	+	+	+	
	anti-Le^bH	−	−	−	−	−	−	−	+	+	+	
	anti-Le^bL	−	+	+	+	+	+	+	+	+	+	
Group O Le(a−b−)	O-Ulex	+	+	+	+	+	+	+	+	+	+	nh nl
	anti-Le^a	−	+	+	+	+	+	+	+	+	+	
	anti-Le^bH	+	+	+	+	+	+	+	+	+	+	
	anti-Le^bL	+	+	+	+	+	+	+	+	+	+	

− agglutination inhibited (specificity present).
+ agglutination not inhibited (specificity absent).

Nonetheless, the alleged presence of Le^b in saliva and its absence from secretions of Le(a−b−) SH individuals was rather disturbing. Fortunately, in a subsequent study a

different anti-Le^b serum was found (Table II) which gives an identical reactivity on red cells and secretions; the saliva of Le(a−b−) SH does not inhibit to any extent such serum; there is, on the contrary, a very slight cross-reactivity with saliva of Le(a+b−) individuals. Therefore, the previous hypothesis about the nature of Le^b specificity can be extended more confidently also to secretions.

It should be emphasized that the two types of anti-Le^b sera (designated as bH and bL in Table II) give an identical pattern of reactions against red cells; the only difference consists in their ability to be inhibited by different soluble substances.

It is probable that the specificity of the two sera is mainly directed against the Le^b antigen, type bL does not cross-react with either erythrocytic H or soluble H; type bH does not cross-react with erythrocytic H but is inhibited by soluble H. As a corollary, erythrocytic H and soluble H are not identical, a conclusion which has been reached also by Watkins and Morgan (1955) in studying the different behaviour of the so-called anti-O and anti-H sera, which is indeed very similar to the behaviour of bL and bH.

Strong evidence in support of the epistatic origin of Le^b specificity has been recently presented on the basis of family segregation data (Ceppellini *et al.*, 1959): out of 131 children born from 38 matings of different Lewis and secretor types, the Le^{bL} specificity was present in the saliva of 95 subjects and absent in 66; the expected ratio was 94·86 : 66·14. The detailed findings in the 38 families were completely compatible with those expected on the assumed hypothesis of two loci, independently inherited but interactive at the phenotypic level.

The transformation of Lewis red cell phenotype IN VITRO and IN VIVO

Stormont (1949) showed that the J antigen of cattle is primarily a soluble substance and it is taken up by erythrocytes on contact with J-positive serum. The same happens

for the R and r antigens of sheep (Rendel *et al.*, 1954). These two animal systems have much in common with the Lewis antigens of Man; and Rendel and co-workers, in fact, suggested that the Lewis factors might easily be taken up by the red cells from the serum. It was already known that the Lewis reactivity diminishes after repeated washing with saline (Brendemoen, 1949) and that the serum shows the ability of inhibiting anti-Lewis sera according to the Lewis red cell phenotype. Later, Sneath and Sneath (1955) showed that it is possible to transform the original Lewis reactivity of the red cells by incubating them for a few days at 35° with appropriate plasma.

A still better proof that the Lewis antigens are not indigenous to the red cells is the fact that in blood group chimeras, while the grafted blood maintains for all other blood groups the original phenotype of the twin-donor, the Lewis antigens, on the contrary, become identical with the Lewis phenotype of the recipient and hence in agreement with his secretor status (Nicholas, Jenkins and Marsh, 1957).

As for J and R-r, one cannot escape the conclusion that the Lewis substances are elaborated by tissues other than those which produce erythrocytes. The first hypothesis would suggest that the Lewis substances which are present in serum, and which are going to coat the erythrocytes, originate as fucomucoids from the same cells which produce the mucoid secretions; but probably the mechanism is not so straightforward and a coupling with some kind of plasma protein is necessary. In fact, the present writer was not able to coat *in vitro* Le(a−b−) red cells with Le(a+) saliva or with purified Le^a substance obtained from human meconium; moreover, two sibs have been observed who showed repeatedly a red cell Le(a−b−) phenotype although, according to the saliva, they were typical non-secretors of ABH and secretors to a high degree of Le^a.

The question of whether or not the Lewis antigens are primarily synthesized by the red cell is of great importance for the interpretation of the many interactions taking place

between the different loci responsible for the final blood group specificities. But not all the pertinent experiments have been done: for instance, while it is certain that in saliva the H and Le^b specificities are closely related (see Ceppellini, 1959), it is not known whether H activity is adsorbed from plasma along with Le^b. Recently Andersen (1958) has shown that a new serological specificity, the Magard factor, can be detected on the red cells of individuals of *A; Se, lele* genotype. That

Table III

SCORES OF H AND LEWIS SPECIFICITIES* IN SALIVA OF INDIVIDUALS WITH DIFFERENT LEWIS PHENOTYPE IN THE RED CELLS (AVERAGE OF 10 INDIVIDUALS FOR EACH PHENOTYPE), ALL OF GROUP O

	H	Le^a	Le^a	Le^bH	Le^bL
Red cells					
Le(a−b+)	61	33	51	59	55
Le(a−b−)	75	0	0	68	0
Le(a+b−)	0	88	78	0	0
Le(a−b−)	0	18	0	0	0

*Measured by means of 2 anti-Le^a and 2 anti Le^b sera

could imply that the secretor and Lewis loci also act on erythrocyte structures. An alternative explanation is that the Magard factor is picked up from plasma and corresponds to a secondary, "epistatic" specificity of the soluble A substance analogous in origin to Le^b.

Whatever may be the origin of the erythrocytic Lewis antigens, there is a strict but not absolute relationship between the Lewis red cell phenotypes and the kinds and amounts of ABH-Lewis specificities which are present in saliva (Table III).

Discussion

Before making any attempt to formulate a general hypothesis which takes care of all the known facts, it is essential to consider the real nature of the so-called "Bombay" (Oh)

phenotype. This rare blood is not agglutinated by either of the two standard typing sera, anti-A and anti-B; thus it should be classified as group O. It fails, however, to react with any kind of anti-H serum and if H is to be regarded as the precursor of A and B antigens, this must represent a metabolic block prior to the formation of H, affecting both red cells and secretions. In fact, all reported Oh bloods are non-secretors of ABH and all belong to the Le(a+b−) phenotype (with one possible exception: see Levine and co-workers (1955) which is also taken into account by the model suggested here; see pattern h, Fig. 2).

The genetic analysis of the very remarkable family described by Levine and co-workers (1955) shows that individuals of the Bombay phenotype must be homozygous for a rare gene, *x*, which as anticipated by Ceppellini (1952) is not an allele of the *ABO* locus; in fact, the proposita (see Fig. 1 of Levine *et al.*, 1955) transmits to one of her children a *B* gene. She also transmits to another child a *Se* gene, which is a further proof that the secretor gene had no characteristic phenotypic effect of its own but only operates by activating the *X* gene to synthesize salivary H; finally, this woman shows a Le(a+b−) erythrocytic phenotype notwithstanding the presence of the *Se* gene, which again suggests that the H specificity is essential for the appearance of Le^b.

If the *xx* genotype represents a metabolic block at a pre-H level, it should be possible to identify as the accumulated product the substrate upon which *X* acts; unfortunately, no investigation has been made as to whether Bombay red cells react more strongly than usual with type XIV anti-pneumococcal serum which, according to immunochemical data, reveals a general reactivity common to all ABH and Lewis antigens (Kabat, 1956). But the saliva of all Bombay individuals (with the possible exception already mentioned) is very rich in Le^a specificity; thus, Le^a substance can be assumed to be the substrate of the $X \leftarrow Se$ synergistic unit in saliva. However, Le^a cannot be the only possible substrate because individuals of *Se; lele* genotype produce H specificity. Thus, the other

alternative precursor of H must be identified with the basic mucopolysaccharide (b.m.), which is endowed with the general chemical properties of blood group substances, shows type XIV cross-reactivity and is the only mucopolysaccharide substance present in the saliva of the double recessive "mutant" *sese; lele*. Conversely, when we observe the genotypes which are blocked at the b.m.→H stage (*xx* or *sese*) the specific action of the *Le* gene seems to consist in the transformation of b.m into Le^a substance. Taking all these facts into account, it is possible to present a working hypothesis indicating the various levels at which the different genes concerned may operate in the formation of mucopolysaccharides with these group specificities.

Because of the lack (as far as is known) of any different effect of the *Se–se* genotypes on red cell phenotype, and assuming that the Lewis antigens are secondarily adsorbed from the plasma onto the red cell surface, only two loci are required for explaining the formation of ABO blood groups· the *X* gene which endows a basic precursor with H specificity and the I^A and I^B genes which transform H into AB and/or B; the *xx* and i^Oi^O genotypes correspond to two different blocks, the first at a pre-H, the second at a post-H level (see Fig. 1).

In the case of the salivary substances the situation is necessarily more complex because the Lewis and the *Se–se* loci are also involved. Nevertheless, all the known facts can be accounted for in terms of the pathways depicted in Fig. 2.

As for the red cells, the I^A and I^B genes can only produce their effects if H has been formed (pattern a, Fig. 2). H can only be found provided X is present and also provided that the appropriate physiological conditions determined by *Se* occur, that explains, as far as secretions are concerned, the identity of the common non-secretor phenotype (*Le; X; sese*. Pattern e, Fig. 2) and the rare Bombay phenotype (*Le; xx; Se*); the existence of a still rarer Bombay Le(a−b−) phenotype (*lele; xx*) is also anticipated (pattern h, Fig. 2).

Both *Le* and *X*(+*Se*) can act on, and perhaps compete for, the same primary substrate (b.m.); their joint action on it

produces the "epistatic" Le^b specificity which is regarded as a secondary immunochemical configuration arising from the insertion of Le^a and H structures on the same molecule.

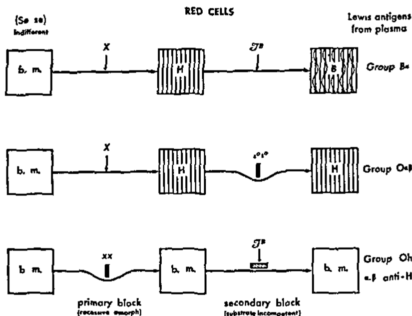


FIG. 1. Scheme of the metabolic patterns which lead to the synthesis of ABH antigens of the red cells It is assumed that the Lewis antigens are acquired *from plasma and that the Se–se locus has no detectable effect on the specificity* of red cell antigens.

This working hypothesis has been derived solely from a consideration of the serological and genetical evidence (Ceppellini *et al.*, 1959). It is gratifying to note that a similar

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FIG. 2. Scheme of the metabolic patterns which lead to the synthesis of salivary mucopolysaccharides with different serological specificities

Symbols · 4 loci (1) *Le–le* (2) *X–x*
(3) *Se–se* (4) I^A, I^B, i^O
le, *x se*, i^O, recessive and amorph

⟶ activity ‑ ‑ ‑ ‑ → inactivity
▮ primary block ▭ secondary block

SALIVA

Le (a b+) SH Les

Le (a b) SH nl

Le (a b+) SH Les

Le (a-b) SH nl

Le (a+b-) nh Les

Le (a b-) nh nl

Le (a+b-) nh Les
Bombay

Le (a b) nh nl
Bombay (?)

FIG 2.

scheme is proposed by Morgan and Watkins (this symposium, pp. 194, 217) as the result of their elegant biochemical studies.

Summary

Since blood group antigens, almost without exception, seem to be inherited as monofactorial Mendelian traits, it was thought that the antigen is the immediate product of a corresponding gene, in accordance with the template theory of gene action. In fact, the blood group antigens are complex macromolecules whose structure has not yet been clearly defined, while their serological specificity derives from relatively simple chemical groupings The question arises whether the unit of inheritance is directly responsible for the synthesis of the macromolecule as a whole or of only certain groupings which determine the serological specificity.

In the case of the soluble blood group specific substances (ABH and Lewis) biochemical and genetical data permit the conclusion that: (1) more than one serological and genetical specificity may be carried by the same macromolecule (e.g. A and B, A and Lewis); (2) the final product, at least in some instances, is reached through a series of metabolic steps (the synthesis of H, genetically controlled by the Xx locus, is necessary for the synthesis of A and B); (3) the interaction of alleles at independent loci may induce a new serological specificity ($X + Se + Le = \mathrm{Le}^b$).

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DISCUSSION

Grubb: A very real problem that has been brought up already here is the question of the control of the distribution of specific substances in various parts of the body.

Most of the difficulties in interpreting the ABH-secretor–Lewis interrelationships arise from our lack of knowledge on this point. There are striking differences in the distribution of these substances.

In Man A substance is present on the red cells, in the saliva and in seminal plasma. Le^a substance is present in saliva but not in seminal plasma, not even in those of red cell type Le(a+). In some primates one finds A substance only in the secretions, not on the red cells. I wonder what kind of control governs these differences in distribution and if there are any informative analogies in other fields.

Ceppellini: Dr. Grubb's remark underlines one of the many fascinating aspects of the problem: why in different tissues the same genetical system shows a different activity and ends up with a different phenotype. The rôle played by the *Se* gene in red cells and mucoid cells is the most obvious example. Quite clearly here we are dealing with the puzzle of differentiation in multicellular organisms. However, a number of different "blood group activity" phenotypes would be more difficult to understand if they were the direct product of one gene; interactions at the cytoplasmic level leave more room for flexible end results.

Burnet: I think it might be relevant to suggest, particularly at a meeting of biochemical geneticists, that there is another system associated with mucoid and red cells, the influenza virus inhibitors and receptors, which would probably bear a good deal of study along similar lines. No genetical work as far as I know has yet been done, but Gottschalk has recently shown an interesting difference between sheep salivary mucin and bovine salivary mucin. Both, as in all such substances, contain neuraminic acid, both contain an acetylhexosamine, for both this appears to be the essential disaccharide complex. But they differ very sharply in the types of influenza virus indicator viruses which they inhibit, this presumably being associated with the relationship of the disaccharide prosthetic group to the protein components of the two mucoproteins. This is a field very closely related to the blood group field, in which useful genetical work might be done, in regard to such differences.

Siniscalco: How do aberrant secretors fit into this genetical scheme?

Ceppellini: There are a few recent reports on aberrant secretions, for instance a group O who secretes A substance or, more precisely, a saliva of a group O individual which inhibits *some* anti-A antisera. In fact, the interesting observations of Beolchini and co-workers (1959, *Vox Sang. (Basel)*, in press) show that "paradoxal" secretions are evidenced only by means of anti-A or anti-B antisera obtained from donors who are "non-paradoxal" secretors. It is quite possible that a number of minor serological specificities related to the major ABH specificities exist and they should be investigated, but I do not think that they will change basically our present ideas.

Morgan: Some time ago Dr. Watkins and I titrated for A, B and

H activity, a large number of saliva specimens from group AB secretor persons and found occasionally a considerable variation in the amount of specific activity in individual saliva specimens. For example, a high A titre did not always go with a high H titre.

Lederberg: Dr. Watkins, are you able to degrade AB substance enzymically and leave A or B activity?

Watkins: Yes.

Lederberg: Does this involve removal of a side-chain?

Watkins: We have only been able to try this with a B enzyme because we have not obtained a sufficiently purified preparation of A enzyme. With the B enzyme, however, there is a removal mainly of free galactose with traces of fucose and *N*-acetylhexosamine as well. There is no evidence for the release of oligosaccharide units, and we believe that the enzymes which destroy the serological activity of the blood group substances function by removing the terminal non-reducing unit of the sugar sequence which determines specificity. After the action of the B enzyme and AB substance one is left with a molecule which has lost its B activity, but which retains its A activity and shows newly developed H serological properties.

HEREDITARY GAMMA GLOBULIN GROUPS IN MAN

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When we speak about gamma globulins the first thing that comes to mind is perhaps their antibody function. In this function gamma globulins are, of course, useful tools for detecting genetical traits, as witnessed in many contexts. Gamma globulins are also of interest to the geneticist in another respect because the modelling of the specificity of the combining sites of gamma globulin by antigen is a directed synthesis of macromolecules exhibiting high-grade specificity. From some points of view this process is related to the problems of gene action. These aspects of the relationship between gamma globulins and genetics are by themselves sufficient to form the subject matter of whole symposia, and these two wide aspects are not the subject of the present paper.

In recent years it has been realized that gamma globulins are useful to the geneticist in still another way. The gamma globulins show intraspecies, hereditary group specificity. We thus have heritable gamma globulin groups. This is known to hold for Man (Grubb, 1956; Grubb and Laurell, 1956) and for the rabbit (Dubiski, Dudziak and Skalba, 1959; Oudin, 1956*a* and *b*; Dray and Young, 1958). The gamma globulin groups in Man are designated the Gm (for gamma) groups.

The serum protein groups we have hitherto heard of in the symposium are demonstrated by electrophoretic methods. The gamma globulin group differences are demonstrated by serological methods. Agglutination-inhibition is used for determining the Gm groups in Man and precipitation reactions for the rabbit gamma globulin groups.

The serological system used to detect the Gm groups consists of:

(1) Rh-positive red cells coated with, but not agglutinated by, certain incomplete anti-Rh.

(2) Selected sera from patients with rheumatoid arthritis, which are capable of agglutinating these coated red cells provided they have been coated with a properly selected human antibody.

(3) The serum to be tested.

The reagents involved, i.e. coated red cells, the rheumatoid arthritis factor and normal serum and their interactions will be apparent from Table I.

Table I

THE REAGENTS INVOLVED IN GM GROUPING AND THEIR INTERACTIONS

Red cells coated	*Agglutination*	*Group*
with human antibody	No	
with human antibody + R A. factor	Yes	
with human antibody + R A. factor + Normal serum	No	Gm(a+)
with human antibody + R.A. factor + Normal serum	Yes	Gm(a−)

Gm(a+) sera thus contain an inhibitor which is not observed in the Gm(a−) sera, or is observed only in much smaller amounts. The anti-Rh used to coat the red cells should emanate from a Gm(a+) person, and this is the main point involved in selecting the proper antibody for coating (Grubb, 1958; Harboe and Lundevall, 1959). As is well known, the agglutination-inhibition test is not without its drawbacks since several different agents might be inhibitory, and another independent technique is, of course, desirable; but, as yet, the agglutination-inhibition technique is the only method available.

The distribution of the Gm groups in some populations is given in Table II. It appears from Table II that 40–61 per cent of Caucasians are Gm(a+). Group Gm(a+) is very

common in people living in the tropics and in those closest to the North Pole.

The inheritance of the Gm groups has been studied in at least 450 families from several different countries (Grubb and Laurell, 1956; Moullec *et al.*, 1956; Linnet-Jepsen, Galatius-Jensen and Hauge, 1958; Lawler, 1958; Makelä and Tiilikainen, 1959; Harboe and Lundevall, 1959). The family

Table II

Frequency of group Gm(a+) in some populations

	% Gm(a+)	n	*References*
Denmark	55 0	1084	Linnet-Jepsen *et al.* (1958)
England	61	133	Lawler (1958)
Finland	65	477	Mäkelä and Tiilikainen (1959)
France	55 7	600	Moullec *et al* (1956) Podliachouck *et al* (1958)
Italy	40 2	125	Lawler (1958)
Norway	60 6	320	Harboe and Lundevall (1959)
Sweden	59 7	360	Grubb and Laurell (1956)
Australia (Whites)	58	100	Kirk (1958)
Asia	100	84	Kirk (1958)
Africa (Dakar)	100	500	Moullec, Fine and Henry (1958)
Eskimos	95	74	Grubb and Laurell (1956)
Lapps (Sweden)	67 3	110	Grubb (1959*b*)

material comprises at least 80 families of mating type Gm(a−) × Gm(a−) and all 237 children from these matings were Gm(a−). The Gm groups in 105 pairs of twins presumed to be monozygotic for other reasons, were found to be the same in both twins (Linnet-Jepsen, Galatius-Jensen and Hauge, 1958; Mäkelä and Tiilikainen, 1959). The family material supports the contention that the Gm^a gene expresses itself in a single dose and that the gene is located in an autosome. No linkage relationship with the "blood groups" or haptoglobin groups has been observed (Grubb and Laurell, 1956;

Laurell and Grubb, 1957; Linnet-Jepsen, Galatius-Jensen and Hauge, 1958).

Until recently Gm(a−) meant only the absence of the Gm(a+) character. Harboe (1959) in Norway has, however, found a serum antithetical to the usual rheumatoid arthritic sera. This serum, also from a patient with rheumatoid arthritis, is thus able to earmark Gm(a−) persons, who could hitherto only be identified by exclusion. The serum also reacts with the heterozygotes. Furthermore, Harboe and Lundevall (1959) have found other sera from patients with rheumatoid arthritis the agglutination activating capacity of which is inhibited only by some Gm(a+) samples. In other words, they have been able to subdivide the Gm(a+) character into two. They have obtained evidence that these newly detected characters in the Gm system are heritable, too. The observations hitherto recorded are consistent with a scheme of three alleles at the *Gm* locus. In the opinion of the present writer, these alleles might preferably be designated Gm^{a_1}, Gm^{a_2} and Gm^{b}. Gene Gm^{a} is not specified as to the subdivision. The relationship between the symbols Gm^{a}, Gm^{a_1} and Gm^{a_2} would be the same as between genes A, A_1 and A_2 in the ABO blood group system.

Before we embark on the question of what the Gm system may perhaps tell us about the mechanism of gene action, the evidence indicating that the Gm characters are contained in gamma globulin should be stated:

(1) In salt-ethanol and electrophoresis fractionation experiments the Gm^{a} character is found in the gamma globulin fraction. This is compatible with the assumption that the Gm^{a} character is contained in the gamma globulins proper or is a contaminant or concomitant of the gamma globulin fraction.

(2) Human antibodies function as the essential element in some indicator systems (anti-Rh + Rh-positive red cells, anti-*Brucellae* + the bacteria, erythrocytes in haemolytic anaemia) (Foz, Batalla and Espacio, 1954; Foz and

Batalla, 1956; Waller and Vaughan, 1956; Grubb, 1956). The antibodies have been highly purified in the absorption and washing procedures used in these tests. Control experiments show that Rh-negative red cells first suspended in anti-Rh, or Rh-positive cells first suspended in normal serum, cannot be used. It thus appears that human antibodies free from other serum constituents are the elements essential for the reaction to take place. The tests using defined, specific antibodies point more definitely to gamma globulin proper as the reactant, than do the other fractionation or diagnostic procedures in which the entire fraction II of human serum is incorporated.

(3) Sera from 18 patients with considerable hypogammaglobulinaemia proved non-inhibitory (Grubb and Laurell, 1956; Vaughan and Good, 1958).

(4) During the first year of life the Gm groups fit in well with what is known about the physiology of gamma globulin in newborns (Moullec *et al.*, 1956; Bronnestam and Nilsson, 1957; Linnet-Jepsen, 1958). At birth they are the same as those of the mother, at 2–4 months the phenotype is Gm(a−) or difficult to determine and later on the genotype becomes manifest.

In the light of this evidence it appears justified to conclude that the Gm specificity is contained in gamma globulin proper.

It is not easy to decide whether the difference between Gm(a+) and Gm(a−) gamma globulin is qualitative or quantitative. The conservative and perhaps correct view is to consider the differences as quantitative, being about tenfold, if we judge it by the haemagglutination-inhibition test. This raises the question whether persons with considerable hypergammaglobulinaemia might phenotypically be Gm(a+) in the absence of the Gm^a gene (cf. Moullec, Fine and Henry, 1958). We have studied this question by doing Gm grouping in the extensive pedigrees showing familial hypergammaglobulinaemia and several cases of lupus erythematosus des-

cribed from Sweden by Leonhardt (1957) and Leonhardt and Larsson (1959). The results showed that there was no correlation between gamma globulin levels and the Gm(a+) character. Several of those persons with the highest gamma globulin levels were Gm(a−). Furthermore, we have encountered a few cases of plasma cell hepatitis in adolescent girls with gamma globulin levels of about 4 per cent which were Gm(a−). It is thus apparent that the Gm(a−) type can coexist with considerable hypergammaglobulinaemia.

It is obvious from the result of the studies of the Gm groups in early infancy and in agammaglobulinaemia that the Gm^a gene cannot express itself, or at least we cannot see the expression, if for physiological or pathological reasons the substrate is not available. This is as self-explanatory as instructive. The gamma globulin molecule is a versatile, domestic type of molecule obeying several different masters. The appearance of gamma globulins in amounts considered normal is dependent upon environmental factors (cf. the studies of Gustafsson and Laurell, 1958, on germ-free animals), physiological status (e.g. in early infancy) and probably also on a sex-linked gene (cf. the studies of Kulneff, Pedersen and Waldenström, 1955, on agammaglobulinaemia) The antigens are masters of the finer structure of gamma globulins as are the *Gm* genes. This is the second example given in this symposium of a molecule being able to serve different masters (cf. the ABO-secretor-Lewis gene interactions).

What is known about the basis of the Gm^a specificity, the way in which the gene Gm^a expresses itself in gamma globulins? We may start with the question of whether the Gm^a specificity expresses itself in or is dependent upon the classical combining sites of antibody molecules. If, when preparing erythrocytes for use in the agglutination test, we try incomplete anti-Rh of the same titre from different donors, we find that only some are useful (Waller and Vaughan, 1956; Grubb, 1956). All these incomplete anti-Rh from different persons have the same specificity and the same reactivity in a number of tests used in Rh serology. If this means that the combining sites of

these donors' anti-Rh are the same, it follows that the Gm[a] specificity is not dependent upon the classical combining site. The fact that antibodies with obviously differing specificities, such as anti-Rh and anti-*Brucellae*, may serve as the essential element in the indicator system, also argues for the independence of the combining sites and the Gm[a] specificity.

Some properties of the Gm[a] character are given in Table III.

As to the absence of Gm[a] specificity from milk, this applies to human milk (3–6 days postpartum) from Gm(a+) women too (Grubb, 1959*b*). The gamma globulins of milk are known to differ somewhat from those of serum (Gugler *et al.*, 1958). Some further leads as to the basis of the Gm specificity might

Table III

PROPERTIES OF Gm[a] SPECIFICITY

Present in	gamma $_7$-globulin, passes the placenta, probably 7 S.
Not present in ·	milk, not dependent upon the classical combining site.
Destroyed by ·	crystalline papain and pepsin, ficin
Not destroyed by	63°C, pH 12 3 at 37°, periodate, sulphydryl compounds, trypsin

(S = Svedberg units.)

be gained by studying gamma globulins in normal serum as compared with those in milk. The indications are that one should include also the various pathological gamma globulins met with in myeloma, in such a study.

The alkali stability of the Gm substance is a somewhat unusual feature. The results of periodate treatment argue against the specificity being contained in the carbohydrate part of the molecule (Grubb, 1958).

The result of the treatment with sulphydryl compounds, which was made by the method of Deutsch and Morton (1957) (cf. Grubb and Swahn, 1958), indicates that cleavable S=S linkages are not important for the Gm specificity. Trypsin attacks native proteins but weakly, and this might explain the lack of effect.

As to the destruction of the activity of Gm[a] substance by crystalline papain, it should be stressed that the specificity rapidly disappears and on relatively mild treatment which

according to Petermann (1946) and Porter (1959), breaks down gamma globulin into molecules with a molecular weight of about 55,000. The Gm^a activity thus disappears upon what is probably the splitting of a very few bonds of the gamma globulin molecule. This evidence may mean that the Gm specificity is highly dependent upon the intactness of the molecular backbone. Other interpretations are, however, possible; but the prospects of finding smaller fragments of the molecule which will show the specificity do not appear too promising.

To recapitulate: the Gm specificity does not appear to reside in the classical combining site or in the carbohydrate part of the gamma globulin molecule; nor does it appear to be dependent on S=S linkages. The unusual alkali stability of the Gm specificity, its rapid disappearance upon treatment with papain and its absence in milk gamma globulin, constitute leads which should be followed up.

Medical genetics is interested not only in the more or less direct action of genes on their specific substrate, but also in the more remote clinical effects of gene action. The chain reaction, gene–gene product–effect on phenotype, appearing as disease symptoms has been worked backwards in tracing many genetical traits in Man. We have, of course, many examples of the detection of genes as a result of a study of diseases. This also applies to the *Gm* genes, which were detected fortuitously in serological studies in rheumatoid arthritis. If the chain reaction has once been worked out, it usually provides a scheme giving insight into the interrelationship between the gene and the disease process. This does not apply to the *Gm* genes and rheumatoid arthritis at our present state of knowledge. The relationship, if any, between the *Gm* genes and rheumatoid arthritis is obscure, and there is no answer to the question, which naturally comes to the fore: what is the significance of the fact that in rheumatoid arthritis there frequently appears a macroglobulin capable of detecting a genetically determined configuration of the human gamma globulin molecule? The studies of the frequency distribution of the Gm groups in

patients with rheumatoid arthritis (Grubb, 1957; Podliachouck, Jacqueline and Eyquem, 1958) seem to show that the distribution is closely similar to that found in normal people. This interpretation cannot be fully accepted until it has been supported by family data on the Gm groups in rheumatoid arthritic patients or until it has been shown that the inhibitor(s) in Gm(a+) persons with rheumatoid arthritis is indeed the same as in normals. This reservation is made because the Gm groups were demonstrated by agglutination–inhibition and the possibility that more than one inhibitor might exist in serum. The versatility of the gamma globulin synthesis process in diseases, as exemplified in the myeloma proteins, is another reason for caution.

There is evidence that rheumatoid arthritis is very uncommon in people in the tropics (Clarke, 1915) and in Eskimos (Hill, 1939). It is of some interest to note that the frequency of the Gm(a+) group is not far from 100 per cent in these populations (cf. Table II).

Our lack of understanding of the relationship between the *Gm* genes and rheumatoid arthritis has its counterpart in our ignorance of the relationship between the rheumatoid arthritis serum factor and the fibrinoid necrosis and other manifestations of rheumatoid arthritis. To complicate matters still more, we have recently observed that fresh sera from a considerable portion of patients with rheumatoid arthritis render rabbits exceedingly sensitive to a subsequent injection of bacterial endotoxin, the LD_{50} then being only about one-thousandth of the ordinary.

The interrelationships in this field are seemingly complicated but rich in data and we may hope that some links making it possible to discern a meaningful pattern may soon appear.

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DISCUSSION

Burnet: Can you ever obtain a suitable incomplete Rh antibody from a person who is known to be a Gm(a−)?

Grubb: I do not think so. Some people have published that this is so, but on closer examination this is seen to be purely a technical fault. If there is too much anti-Rh there will not be any inhibition because of the great excess of anti-Rh. If you first absorb out the anti-Rh you will find the sera to be Gm(a+) I have not seen any and I have studied some 50 of them.

Siniscalco: Dr. Grubb, have you any information on the inhibition that some human sera or plasma seem to exhibit on the agglutination of latex particles by rheumatoid arthritic sera? Olivelli has reported that about 60 per cent of the sera from normal people show such inhibitory effect and he thinks that they might be Gm(a+) sera and that this method could therefore be another way of performing the Gm-typing [Olivelli, F. (1958). *Riv. Emoter. Immuno-emat.*, 5, 425].

Dr. Adinolfi in our Department has repeated the test on a selected sample of Gm(a+) and Gm(a−) individuals and found that while he was able to confirm the existence of the phenomenon, there was no correlation with the Gm phenotypes. We do not yet know whether this effect is a stable one and whether it is genetically controlled.

Secondly, we have been extending the population data on the Gm groups in Italy and found, as shown in Table I, that not only in the district of Ferrara but also in those of Naples and Salerno and in Sardinia, the incidence of Gm(a+) individuals seems to be of the order of 40 per cent.

Finally, concerning linkage investigations we have been doing a large family analysis and found no obvious linkage relationships with the loci listed in Table II, but it would be worth while to get some more informative families for the comparison with the P blood group system, since the sum of the scores in this case is more than twice its standard error.

Grubb. I have no personal experience of the latex-inhibition test but I would guess that the possibility of its being used in this context depends on what you put on the latex particles. I am not sure that it would work in the same way as the Gm. There are some other inhibitors which work in the latex fixation test i.e. some complement components are inhibitory in the latex fixation test but not in the Gm test. It is also said that some Pneumococcus polysaccharides are inhibitory in the latex fixation test but not in the red cell test. The frequencies of the Gm groups in Italy are the lowest in the world. We have these very large and striking differences between different populations; in Africa we have about 100 per cent. One

Table I (Siniscalco)

POPULATION DATA (SAMPLES OF UNRELATED PEOPLE)

Localities	*No. of cases*	Gm(a+)	Gm(a−)	*Estimated gene frequencies* Gm^a	Gm	S E.
Cologna (District of Ferrara)	185	70 (0 378)	115 (0 622)	0 21	0 79	±0 023
Atena (District of Salerno)	73	34 (0 465)	39 (0 535)	0 27	0 73	±0 040
Galtelli (District of Nuoro)	102	32 (0 314)	70 (0·686)	0 17	0 83	±0 028
Naples	84	34 (0 405)	50 (0 595)	0 23	0·77	±0 035
Totals	444	170 (0·383)	274 (0·617)			

Heterogeneity in Gm-group frequencies for different centres $\chi^2 = 4 \cdot 25$, d.f. = 3, $P > 0\ 20$

Table II (Siniscalco)

LINKAGE BETWEEN GENES FOR Gm-GROUPS AND OTHER AUTOSOMAL LOCI

Test characters	*No of informative families**	$\Sigma(\lambda)$	Σ(V)
Haptoglobins	16	−3 055	63·00
Thalassaemia	10	−5·778	20·00
ABO blood group	11	−7 000	28·75
MNS blood group	12	−1 556	69·28
P blood group	10	+5 110	7 12
Rh blood group	10	+4·333	37 15
Lutheran blood group	—	—	—
Kell blood group	5	+0 333	10 11
Duffy blood group	11	+7 778	46·37

* Certain families only

wonders why we have these differences; if we ask why we have different blood groups or Gm groups, that is hardly a meaningful question in our present state of knowledge, but I wonder if someone has any idea why we have these different haptoglobin groups.

Smithies: I can make a suggestion as to a possible selective mechanism although I cannot really offer any proof of its correctness. The work of Laurell and Nyman and of others seems to establish that the presence of circulating haptoglobin will prevent the loss of haemoglobin into the urine when it is liberated into the circulation, e.g. by haemolysis [Laurell, C. B., and Nyman, M. (1957). *Blood*, **12**, 493]. If an individual has no circulating haptoglobin then haemoglobin can pass the glomerular membrane and be excreted. Loss of haemoglobin can thus occur in individuals lacking haptoglobin. This could conceivably introduce some slight selection between the different types. Thus, the large molecular weight type 2-2 haptoglobins do not readily pass the glomerular membrane when it is damaged Hence, individuals who are type 2-2 might retain their haptoglobins under circumstances in which individuals who are type 1-1 (which is the low-molecular weight type of haptoglobin) might lose their haptoglobin. Unfortunately even if this type of selection were to occur it does not account for the presumed advantage of being a heterozygote.

Wright: Dr. Grubb, instead of using rheumatoid arthritis sera, can you substitute sera from patients with other collagen disorders, rheumatic fever or dermatomyositis or Still's disease, with the same results?

Grubb: With rheumatic fever sera you cannot do it. With several of the other collagen diseases we have not been able to do it. We have, however, been able to do Gm grouping with serum from girls with plasma cell hepatitis. Very infrequently you can find the same thing in an apparently normal person, in 1 in 2,000 or so; but this is not true of all collagen diseases; this specific example of plasma cell hepatitis is the only one that I know of.

Kalmus: This is not clear to me. Do not the rheumatoid arthritis sera contain antibodies against traumatically released substances acting in the manner of foreign antigens? Perhaps somewhat in the way in which, following damage to one eye, after a while the previously healthy eye is attacked in sympathetic ophthalmia by antibodies induced by the damaged tissue of the first eye.

Grubb: I am careful not to use the word antibody for this substance in rheumatoid arthritis serum. So far, it is not known whether it is an antibody or not, and it is very easy to get mixed up in talking about autoimmunization in this connexion.

Ceppellini: When you say that rare normal sera give this effect, I think your factor is the so-called Milgrom factor. Milgrom stated that in some normal people you find a factor which agglutinates Rh-sensitized cells but is not inhibited by any serum, and he claimed that one could use this Milgrom factor for doing a Coombs test without washing the cells. Another point is that many of us feel that the rheumatoid factor is an antibody or at least is reacting against denaturated globulins, or maybe globulins that have been denaturated in a specific way just because they unite with the antigen

Grubb: The Milgrom type of factor is not inhibited by gamma globulin in solution and thus differs from the rheumatoid arthritis type. But in a few normal sera you find a factor which reacts exactly like the one in rheumatoid arthritis and which is inhibited by gamma globulin in solution. The question whether it is an antibody is largely a matter of semantics. If you inject gamma globulin into a person possessing rheumatoid arthritis factor you will find that the half life time is the same as in normals. So in this respect it does not function as an antibody.

Brenner: Have you tested myeloma protein for Gm(a+) and Gm(a−)?

Grubb: No, we intend to do that and are collecting some data.

Harris: Do you find the inhibitor is in myeloma plasma, irrespective of whether it is part of the myeloma protein?

Grubb: We have had two patients with myeloma, and these had protein in the urine. Their plasma did not inhibit but they might have been Gm(a−); we must have more material before we can make a statement about this.

Harris: In myelomatosis does the myeloma protein itself behave as an antibody?

Grubb: The sera of these patients are often poor in antibody, not infrequently the anti-A and anti-B are very low; these cases might physiologically be more or less like agammaglobulinaemia, in spite of a hypergammaglobulinaemia

Smithies: Dr Grubb, you said that about 1 in 2,000 normal persons showed the same agglutinating factor as is present in a much higher proportion in persons with rheumatoid arthritis. Do you think there is any possibility that the rheumatoid arthritis condition is selecting these people from the normal population?

Grubb: It is an important question but, unfortunately, the answer is difficult to obtain.

Smithies: Do you have any proof that the same protein is inhibitory in, say, a Negro population, in which such a high proportion of sera are inhibitory, as in a European population?

Grubb: I cannot be sure of that. There is unpublished evidence that there are some different types in Negro populations.

Burnet: You mentioned other types of rheumatoid arthritis sera anti-A with entirely different specificities. Is your Gm(b) the same as Gm(a)?

Grubb: Yes.

Burnet: And that is produced with the same type of indicator Rh incomplete serum.

Grubb: You must use anti-Rh produced in Gm(a−) individuals.

Burnet: So that this a and b do represent more or less homologous types of antibody?

Grubb: Yes.

Lederberg. The sera from your rheumatoid arthritis donors show both the macroglobulin antibody and a globulin with which it reacts?

Grubb: You might find this and it appears as a prozone phenomenon.

Wright: From one of your tables I got the impression that the frequency of Gm(a+) was higher in those countries where rheumatoid arthritis may be infrequent, e.g. Africa. Is there any correlation between the distribution of these Gm types and the frequency of rheumatoid arthritis?

Grubb. There is evidence that in the area of the Equator rheumatoid arthritis is very infrequent, and you can also find statements that it is infrequent in Eskimos; I don't know more than that and I don't know what it means.

Brenner: Am I correct in saying that amongst rheumatoid arthritis cases the distribution of Gm(a+) and Gm(a−) is very similar to that in the general population?

Grubb: Yes.

PANEL DISCUSSION

Montalenti: Prof. Penrose said earlier that tissue culture is the new star in the sky of human genetics, and whether it is a star of prime magnitude or just a starlet we are now about to hear. The approach to genetical problems by tissue culture is only at the very beginning, and so we have arranged a discussion by a panel composed of Prof. Luria, Prof. Pontecorvo, Prof. Cavalli-Sforza, Prof. Eagle and Prof. Lederberg. I have asked Prof. Luria to be the moderator.

Luria: It is good that we have Dr. Eagle here, otherwise this would almost be a panel of inexperts since some of us have not done anything with tissue cultures. However, Dr. Eagle is a real expert and we shall fall back on him for factual information.

As is well known, the tissue culture approach to biological problems has a long history of successes and of heartbreaks. Many hopes were placed in this approach in connexion with the matter of differentiation and these hopes have not been fulfilled. In recent years it is the work on viruses that has greatly stimulated, and gained from, the tissue culture approach. With the coming of the new and simplified methods of tissue culture, especially those which allow prolonged cultivation of cells from a great variety of individuals—including adult individuals—the possibility has arisen to use tissue culture for genetical studies, especially in connexion with the subject of this symposium, i.e. human genetics

Of the approaches that come to mind, the first one is the use of cells derived from individuals with known genetical background, with markers recognizable at the cellular level. This should permit, for example, the recognition of phenomena of recombination within diploid lines. This topic will be discussed first, in connexion with the general problems of the use of tissue culture in human genetics, by Prof. Pontecorvo. Next we thought that our discussion should cover the second type of approach. This is the use of lines of cells in tissue culture for genetical studies analogous to those employed in the genetics of micro-organisms. This includes the study of mutational and recombinational events and possibly also of infectious heredity in cells already established in tissue cultures. This approach also provides new ways of attacking the problem of gene action, including regulation and different expression of genes in different cells, either derived from different tissues or submitted to different environmental conditions. One of the things to be discussed, both as a possible source of error and as a field for genetical analysis, concerns the disturbing events that can occur in the process of establishment of cell lines in tissue culture. So we begin with Prof. Pontecorvo. We hope to keep the discussion as informal as possible; this should be more fruitful than a series of formal presentations

Pontecorvo: I am sure that some of you must have felt like me, in the last three days, a growing admiration for the success of the chemist and a sense of frustration for the genetical approach in human genetics. It seems to me that it is a really sad moment when the chemist has to tell the geneticist e.g. that haemoglobin G must be determined by a mutation in "cistron" α rather than in "cistron" β, on purely chemical grounds. The geneticist can do nothing, or very little, about it in the case of Man, while in the case of experimental organisms, the geneticist was many years ahead of the chemist in showing what sort of ultimate structure and function the genetical material must have. We must find short cuts to genetical

analysis in Man. The present methods are quite inadequate, especially when we come to problems like those which we are debating: fine genetic structure and gene action For these problems we need extremely high genetic resolution if we are to get anywhere.

About five years ago, when I was just beginning to become senile (in Prof. Penrose's definition), I thought that some work which we had done in the previous ten years on somatic segregation in lower organisms might give a clue as to how to overcome the impossibility of experimental breeding and the difficulty of breeding in large numbers in the case of Man. How and why could somatic segregation and recombination be used in genetical analysis in Man? First of all, there are two ways of using it. One is to study somatic segregation in the soma and the other is to study it in cultures derived from the soma.

The work with *Aspergillus* and other lower organisms has given quite clear ideas both of the techniques that can be used, and of the processes at work. (Incidentally, in lower organisms which have no true soma we have to use the term "mitotic" rather than "somatic" segregation.) These techniques for handling mitotic segregation in the analysis of the genotypes of diploid cells multiplying clonally are so easy, and have such advantages over analysis based on sexual reproduction, that we use them now as a routine in a number of species of lower organisms. The location of the centromeres, the determination of the order of loci, and the assignation of an unlocated mutant to a linkage group are quite simple.

Now, what the *Aspergillus* work has led us to realize is that there are at least three processes of somatic segregation and recombination. In the case of one of these, i e. somatic crossing over, we do not know whether or not it occurs in mammals, including Man. We know that it occurs in certain insects. We know that it possibly occurs in certain plants, but we do not know about its occurrence in mammals. The other two processes are "non-disjunction" and what I have called "haploidization". The former could, in fact, be only a different result of the same process which underlies haploidization.

Consider a cell heterozygous for two linked genes· $\frac{AB}{ab}$. The cell divides mitotically and starts a clone of cells. At a certain point—this is a rare event—one of the cells of this clone gives origin not to two daughter cells with a genotype like its own but to two daughter cells of one of the following types. One is homozygous *AB*, the other is homozygous *ab*, or alternatively one is still heterozygous *A*/*a*, but homozygous *B*, and the other is still heterozygous *A*/*a* but homozygous *b*. This is what somatic crossing over produces. The details of it are now perfectly clear. It was discovered by Stern in *Drosophila*

in 1936, and the amount of information we have now added makes Stern's interpretation certain. Somatic crossing over permits the identification of the location of the centromeres and of the sequence of loci in one arm of any one chromosome. As I said, we have no clue as yet as to whether or not somatic crossing over occurs in vertebrates and in particular in Man.

A second process of somatic segregation is haploidization. Consider a cell heterozygous for a number of markers on a number of chromosomes. Such a cell, by an accident of mitosis, gives origin to a cell which has lost one of its chromosomes. This puts a premium on further losses, so that in the clonal line derived from that cell, eventually either one or the other member of each of the other pairs of chromosomes is lost until finally a completely haploid set is attained. This haploid set may of course be made up of any association between the members of the various chromosome pairs There is, therefore, recombination between whole chromosomes, and different recombinant sublines arise in the clone.

Brenner: Why do you say there is a premium on further losses?

Pontecorvo: This is an experimental fact in *Aspergillus nidulans* (which has eight chromosomes) and I believe it to be likely in other cases. If you isolate segregant cells only a few nuclear generations after segregation for one marker has occurred, you can follow the occurrence of further losses until the complete haploid set is attained. On the other hand, another compensatory process can occur, namely non-disjunction, about which I shall talk in a moment. When a loss of one member of a chromosome pair takes place there is a premium put on duplication of the now haploid ("monosomic") chromosome. This produces homozygosis for one whole chromosome, but all the rest remains heterozygous

Luria: Would you think that there is a premium on further losses, or that there may be in such lines a mechanism that continues to favour losses?

Brenner: Could they be clonal?

Pontecorvo: They could be both, i.e. accidental losses selected and further losses favoured by the same cause which produced the first loss My guess is that the most common situation is simply one of selection, and not that *of some permanent damage in the cell which is* transmitted in the cell lineage and goes on producing chromosome losses. A third possibility would be that the imbalance, resulting from one accidental loss, favours further losses.

Lederberg: It should be stressed that you are culturing an organism in a nearly normal habitat, corresponding to the one in which its chromosome complement has evolved The same consideration does not necessarily apply equally to tissue culture, or to transplanted

tumours whose new environment may well select for, as well as induce, karyotypic innovations.

Pontecorvo: Closing this parenthesis, the third process of somatic segregation is "non-disjunction". The first step is the same as in haploidization, namely one member of one chromosome pair is accidentally lost, and the compensatory process is simply that of non-disjunction of the monosomic chromosome. Non-disjunction is known to occur in every organism which has been investigated cytologically and/or genetically. I repeat that all three processes of somatic segregation are rare, of the order of once in a few hundred or thousand cells.

There is no doubt that while we do not know whether or not somatic crossing over occurs in Man—and it is possible that it does not—these other two processes must occur: they have been seen in careful cytological work with organisms of all kinds, including tissue cultures of Man.

Even if somatic crossing over did not occur in Man, these other two processes of accidental—or perhaps non-accidental—loss of chromosomes and compensatory processes like non-disjunction would be quite enough to carry out genetical analysis via somatic segregation to a very considerable extent. They permit individual genes to be assigned to individual linkage groups. They do not permit, as mitotic crossing over does, the establishment of the linear order of the genes on one chromosome.

Somatic segregation, therefore, could be used for genetical analysis in Man even if limited to these two processes. It could be used in the soma itself, or in tissue cultures. In the soma, somatic segregation would lead to small or large clones of tissues differing in genotype from the rest as a consequence of one of the processes mentioned above. That this expected consequence of somatic segregation could be tested relatively simply, occurred three years ago to Dr. Goudie in Glasgow. He searched for it in the red cells of AB individuals. The matter was then taken up independently by Atwood who developed a far more refined technique, using isotopic chromic acid, and confirmed one of Goudie's conclusions. There is no doubt that the erythrocytes of individuals heterozygous for blood group antigens always seems to carry a small proportion of cells which have only one of two possible alleles. There is, of course, no proof at all that this is due to somatic segregation. But it simply means that somatic segregation is not excluded at least as a possible source of some of the unexpected cells.

The limitations of the study of somatic segregation in the soma are very great. We can see some of them already in the classical example of the study of somatic segregation: Stern's work with *Drosophila*.

In Drosophila there were only 4 or 5 markers which lent themselves to the study of somatic segregation in the soma, because the markers have to be of a very special kind. First, they have to be detectable in small patches, representing only a small proportion of the total body. This proportion could be small when dealing with fluid tissues like blood but in the case, e g. of mosaic patches in the skin one needs patches of quite a substantial size to distinguish them from the background. An exception is the case of the bristles in Drosophila in which individual bristles of recessive type in a background of dominants can be detected. A second and more serious limitation is that any recessive allele which has non-autonomous action would not be detectable in a segregant patch because the diffused substances from the surrounding tissues (heterozygous and phenotypically dominant) would mask the mutant genotype. So it seems to me that the scope of the study of somatic segregation in the soma is rather limited. However, it has some interesting possibilities, especially with the blood. I do not know whether Prof. Ceppellini has carried out the plan which he was considering in 1958, to study the formation of doubly homozygous red cells in individuals heterozygous at two linked loci both determining antigenic properties. These are very interesting possibilities and I think it would be very good if Ceppellini and others with the same kind of skill will try them out

Ceppellini: No, unfortunately I cannot. I was thinking of the MN, Ss, Hunter, Henshow, Mi and Vw chromosomes (cf. Race and Sanger, 1959; Blood Groups in Man Oxford: Blackwell). Probably some of these factors are not too closely linked; in any case it would be interesting to follow the behaviour of other related specificities in cell populations selected for a change in one of them (for instance M cells separated from MN blood) However, disappearance of an antigen up to now has only been observed for the ABO groups, of course it is possible that the change only corresponds to a developmental disturbance of the phenotype.

Pontecorvo: Yes, I only say that the evidence so far is not against somatic segregation as the cause of some of the aberrant red cells identified.

Neel: Dr. Atwood a few weeks ago said that he has now observed a striking increase in the proportion of these exceptional cells in individuals who have received ^{32}P for therapeutic reasons. I believe he feels that the increase is more likely to be of a mutational origin than from somatic crossing-over with segregation.

Pontecorvo: I do not think that holds at all, because radiation and many mutagens affect at the same time abnormal segregation, crossing-over, mutation, and development of the phenotype.

Harris: What do you think may be the difference in relative frequency between mutation and somatic segregation?

Pontecorvo: I do not know. It depends on the organism, on the locus, on external conditions and on the genotype.

Harris: In the Atwood situation, for instance, is the general idea that segregation of this sort occurs, say, ten times more frequently than mutation?

Pontecorvo: I do not think you can use this sort of argument; in *Aspergillus*, for distal genes somatic crossing-over is of the order of 10^{-4}, haploidization is of the order of 10^{-5}, and non-disjunction is rarer than either of them, because it requires coincidence of two events.

Kalckar: Can you in this case distinguish between a loss of a gene and the loss of the expression of a gene? I presume that experiments on ^{32}P incorporation are meant as a tool for distinguishing between these two possibilities.

Neel: I do not know; but one would assume that the action of ^{32}P is on nucleated cells.

Kalckar: Differentiation often brings about a loss of expression of a gene.

Pontecorvo: You cannot distinguish loss from mutation when you are dealing with a single marker. You can distinguish loss from mutation when you are dealing with a number of markers, some of which, at least, are linked.

Well, this is enough as to somatic segregation in the soma. It is clear that one can think of doing the same sort of analysis in tissue culture. One could establish cultures from suitable tissues of healthy donors. These donors differ from one another in genotypes. The formation of segregant clones in tissue culture would be a means of detecting heterozygosis from any particular marker which can be detected.

The problem can be approached in two ways One is to start from individuals who differ from one another in genetically determined features and try to see whether these differences can be identified at the cell level. The other way is the opposite one, i.e. to establish cultures from a large number of individuals and submit them to all possible screening tests in an attempt to identify differences between cultures, then go back to the individuals and see whether the effect of the genetic difference—discovered at the cellular level—is identifiable in the individual as a whole either as a difference in morphological or physiological characters or in any other kind of feature.

As some of you may know, in my laboratory we are attempting both approaches. In respect of the second, we are testing for differences between cultures from individual donors in reactions to a

spectrum of viruses, we are testing for differences in antigens, enzymes and other proteins, and we propose to test for differences in reaction to drugs. The obvious thing is to begin with a few clearly identifiable markers. After a difficult start I am quite confident that things will become feasible. But I want to give you an idea of my estimate of the pace of work, because I think there is too much expectation for what the approach via tissue cultures will do. I would be extremely happy if within the next ten years we could begin to get somewhere.

Luria: I do not know if this is appropriate, but may I ask Prof. Penrose for the definition of senility in a geneticist?

Penrose: This is Pontecorvo's interpretation of an aside which was made for my own amusement.

Harris. Could we have Pontecorvo's interpretation?

Pontecorvo: I thought that the very reasonable idea was that when a general geneticist becomes senile he turns to human genetics.

Luria: One of the ways to use tissue cultures for genetic work is to handle them in the same way that unicellular organisms are used in genetic studies. Prof Cavalli-Sforza will tell us something along these lines.

Cavalli-Sforza. It is very difficult to speak about a field of work which is only at its beginning. It is true that the rate of development of bacterial genetics has been very high, at least after an initial lag. Cell genetics has the advantage of having a great deal in common with bacterial genetics, and therefore many ideas and methods can be transplanted directly from one field to the other. Even so, the development of cell genetics is bound to be slower, if nothing else because cells reproduce so much more slowly than bacteria (about 60 ×). However, this is not the only, or the major, cause of delay. We are probably still at a stage of development of the field which, in a growing bacterial culture, would be defined as the stage of "positive acceleration". In a short while, however, progress is likely to become more satisfactory.

Part of the subject I want to discuss has already been dealt with by Prof. Pontecorvo, in the sense that he has already indicated the approaches that one can follow in the search for markers. Markers or heritable differences between cell lines must be obtained in order to start any genetical investigation, and the two kinds of markers that will be useful in this field are those which can be detected in the donors as well as in the cell lines derived from them; and those which can be detected only in cell cultures. In our laboratory we are trying both these lines of approach to secure markers; naturally it is the first type which is more interesting.

Results are not easy to obtain because of difficulties, first of all

connected with the techniques themselves, which need further refinement. This is the experience of our laboratory, which can be extended to most laboratories that I know of. The preparation of media is still somewhat troublesome; media are fairly well defined but they need great care, and they are not yet optimal. The maintenance of the lines is easy only if they are the so-called "stabilized" lines, but it is clear that "stabilized" lines should really not be employed for most genetic work, as I shall mention later. On the other hand, the maintenance of freshly isolated cultures is more difficult. Fortunately, a certain amount of work can be done on "primary cultures" without carrying them further, or carrying them only for a few transfers. Thus, human cytology has been made much easier by the use of primary cultures and it is likely, for instance, that cases of non-disjunction (now known for two chromosome pairs) will increase in number, and more pathological syndromes in addition to mongolism and sex disturbances may prove to be the result of an aberrant karyotype. Such non-disjunction cases could be employed for the purpose of the study of linkage; non-disjunction may in fact prove to be the simplest way of assessing linkage groups for markers detectable only in the donor, by a study of the carriers of specific non-disjunctions, for markers detectable both on the donors and on the cell lines derived from them, and also for markers detectable only on cells.

When, however, a primary culture is established and carried further, there is clearly a great deal of selection presumably because the conditions of culture are not optimal, and also because inevitably cells that can reproduce faster are at an advantage. One thus creates an artificial unicellular organism which has very little connexion with the tissue of origin. What especially shows the peculiarities of the mode of growth of the cell lines which are usually employed, the so-called "stabilized" lines (i.e. lines that have resisted in culture long enough to be distributed to other laboratories) is their cytological investigation. Dr. Chiarelli in our laboratory has examined cytologically some "stabilized" lines of human origin, both pathological and normal. They all show an abnormal chromosome set: chromosome numbers are highly variable from cell to cell, e.g. within a range, for one of our lines, of from 52 to 78. Also, extreme cases were found in some lines, e.g. a cell with 21 chromosomes (an aberrant haploid) and one cell with 102. It is clear that some of the standard chromosome pairs are represented three and four times in the cells, there being a great variation from cell to cell. Cloning somewhat reduces but apparently does not check the variation completely; work is being done with a view to measuring the amount of variation in chromosome numbers taking place in ordinary culture conditions.

It is clear that with such an extreme chromosome variability some types of genetical researches e g. mutation and segregation studies may be worthless, or their results may be valid strictly only for the line on which they were obtained, and wide discrepancies may be expected for different stabilized lines.

Montalenti: What is known about the variation of chromosome number in normal somatic cells?

Cavalli-Sforza: Most tissues show an unusually low frequency of polyploid cells, but most of the work has been done by the method of measuring the diameter of nuclei or the total amount of DNA per nucleus, so it can give only very rough information. It will show that there are diploid, tetraploid or octoploid cells. Chromosome counting is best done in tissue culture when it is easier to find mitoses In primary cultures usually most cells have the standard chromosome number, but not strictly all. Already at the first transfer there may be abnormal cells which have lost a chromosome or have an extra chromosome.

Montalenti: Is there a laboratory technique for getting stabilized lines?

Cavalli-Sforza: I understand that Dr. Eagle has something to say about that, so I will leave the answer to him.

It may be interesting to hear shortly of the experience we have had so far in our laboratory in the search for markers of cell cultures. Dr. De Carli who has been working on this has obtained some markers which have apparently arisen by mutation in our lines, for resistance, e g. to sarcolysin and to atabrin; "biochemical" markers, e.g. for arginine independence. All of the markers found seem to have a slower growth rate than the parent type, and they have also a lower efficiency of plating. This has been examined particularly for the sarcolysin-resistant marker, for which a mutation rate of about 10^{-8} was found, but reconstruction experiments have shown that the efficiency of plating of this mutant is very low and the mutation rate must have been underestimated probably by more than one order of magnitude. This merely indicates some of the difficulties met with in finding good markers. The main requirements of a good marker: low reversion rate, growth rate and plating efficiency comparable to that of the wild type are not frequent—even in bacteria—and a number of markers need to be examined before a good one is found. One particular type of variation which seems well suited for genetical analysis, again borrowing techniques from bacterial genetics, is that of the sugar-fermenting abilities of the cell lines. Fermentation mutants are easily found and commonly employed in bacteria. A survey of the fermentation abilities of our cell lines, substituting for glucose in the medium with the sugar under test, has

shown that our human lines can ferment galactose, fructose, mannose and maltose; they cannot ferment arabinose, xylose, lactose, sucrose, raffinose or mannitol. Some differences have been found between established lines, e.g. for maltose, which two lines can ferment while one does not—or does so only under peculiar circumstances. It is likely that mutants for sugar fermentation, acquiring new abilities or losing old ones, will prove useful.

In conclusion, the experience of the first two years has shown us that the field, however promising, demands the solution of a number of technical problems. One can agree with Prof. Pontecorvo that it will take a fairly long time before these things will be developed in full, although I would tend to reduce the number of years somewhat; but this question is entirely subjective.

Luria: An interesting point concerning technique is the remarkable finding reported from the Toronto laboratory that there is a very serious danger of contamination from established lines on to lines which are in the process of being established. It appears that a number of lines thought to have been established directly from normal tissues consisted instead of cells of an established line of a single tumour from mice, which was carried in the same laboratory. It is remarkable how these cells, which are supposedly difficult to keep, once established can jump from one dish or flask to another.

Eagle: Such contamination is not altogether surprising. The frequency is perhaps no more than you would get in a bacteriological laboratory, the difference being only that in the bacteriological laboratory it is readily recognized, while in the case of the animal cultures there are so few readily recognized markers that it is just not detected. With hundreds of people carrying out thousands of culture transfers, it is to be expected that a careless technician, in doing his routine transfers, will occasionally use the same pipette for two cultures. If there is a selective advantage for one, the contaminating cell, a HeLa cell for example, could be replaced by L, or the converse.

Luria: The reason for mentioning it is the fact that the Toronto group had the impression that the real danger of contamination was coming not from bad technique but from too fastidious a technique: with elaborate methods employing automatic pipettes and transfers under hoods, everything goes slowly, the flasks and the tubes stay open a long time and the pipettes spray cell suspensions around This creates more danger of contamination than the usual quick bacteriological operations.

Eagle: Prof. Pontecorvo and Prof. Cavalli-Sforza have outlined some of the uses to which cell cultures could be put. I shall summarize briefly the present state of the art, and point out what I believe

to be some serious limitations in the immediate usefulness of cell culture for the exploration of some of the problems which they have outlined. The use of the word tissue culture in this connexion is a misnomer, for we are not discussing explant cultures of organized bits of tissues, but cultures of dispersed cells. The point is not merely semantic. These two types of cultures differ in so many respects, and particularly both with respect to the rate of growth and function, that they must be clearly distinguished. Cells have been cultured from a wide variety of tissues deriving from Man and animals, both normal and malignant, adult and embryonic.

There are at least four aspects of these cell cultures which are relevant to the theme of this symposium The first is the extraordinary metabolic uniformity in all the mammalian cell cultures which have been so far examined. Whatever the host of origin, and whatever the tissue of origin, all cell lines so far examined have proved to require essentially the same nutrilites for growth. These have been almost completely defined. I would disagree with Prof Cavalli-Sforza, in that what has impressed me is not the complexity of the medium, but its simplicity. It has developed that all mammalian cells require at least 13 amino acids, and the same 13. In contrast, *Man requires only 8 essential amino acids for nitrogen balance in* short-term feeding experiments. Additionally, these cultures require arginine and histidine, they cannot hydroxylate phenylalanine to tyrosine, they require glutamine, and they cannot use methionine adequately for the biosynthesis of cysteine. There are some interesting aspects of the glutamine and cysteine requirements which we have not time to discuss here

These cell lines all require carbohydrates A number of hexoses, and also ribose, will substitute for glucose. There are some interesting differences between the various substrates with respect to the amounts metabolized per unit growth and the amount of glycolysis. In this respect, also, there is no important difference between any of the human cell lines which have been so far examined.

Only six ionic species have been found to be required, and are required again by all of the cell lines so far examined. Almost certainly, additional trace elements are necessary which are present as contaminants in the other components of the medium, notably the serum protein. All the cell lines so far examined require 8 vitamins, although there have been reports of variants which do not require one or another of these 8 vitamins In general, cofactors will substitute for the parent vitamins although there are important quantitative differences in their relative activity.

Over and above these 28 factors, a small amount of serum protein is required. One can use dialysed serum, or one can precipitate the

proteins with ammonium sulphate and use the redialysed protein. The function of the serum protein has not yet been clearly elaborated. In cultures in which the cells are growing on a glass surface, it has been reported to promote the adhesion of the cell to the glass surface. Obviously that is not the function of the serum protein in suspension cultures in which the serum protein is as necessary as it is in monolayer cultures. (*Parenthetically, they are proving increasingly useful in studies on virus formation and the metabolism of the cell as affected by viral infection.*) I suspect that the primary function of the serum protein is to act as the carrier for one or more components of small molecular weight, which are bound to the protein and which are slowly released into the medium. Using labelled serum protein, we have been able to show that at least 95 per cent of the cell protein derives from the free amino acids of the medium rather than the serum protein. Of the 3 to 4 per cent of the cell amino acids which do derive from the serum protein in the medium, recent data would suggest that a large part reflects the utilization of amino acids bound to the serum protein, rather than the breakdown and utilization of the serum protein itself.

The metabolic uniformity in their nutritional requirements is not the only respect in which these cultured cells are alike. They are similar also in the precursors used for the biosynthesis of the non-essential amino acids. Glucose supplies the carbon for the biosynthesis of alanine, serine and glycine, and glutamine for the biosynthesis of aspartic acid, proline and asparagine, with very little interrelationship between these two families of amino acids. The precursors used for nucleic acid synthesis are similar. The cellular amino acid pools are similar, both quantitatively and qualitatively. Finally, there is active protein turnover which proceeds at the same rate in all the cell lines examined, approximately 1 per cent per hour. Relative to the rate of growth, the turnover rate is much larger than in either bacteria or yeast. There is the further qualitative difference that in animal cells, and unlike either bacteria or yeast, the turnover process is the same whether the cell is growing or resting.

Although I have stressed the metabolic uniformity of these cultured lines, it is necessary to point out that minor differences have been noted in certain cell lines. For example, all the human cells so far studied cannot synthesize inositol from glucose in amounts adequate for growth. On the other hand, a mouse fibroblast does. A rabbit fibroblast strain is unable to make sufficient serine from glucose for growth, and the cells will not grow in the absence of added serine. Monkey kidney cells in primary culture have a specific requirement for glycine, which is not satisfied by serine. In this case the metabolic block apparently consists in the reduction of folic

acid to the metabolically active cofactor. On the addition of folinic acid, the glycine requirement is eliminated. The Walker carcinoma is unable to synthesize asparagine from glutamine and requires both glutamine and asparagine. Finally, in these established cell cultures the requirements for the growth of a single cell are almost precisely the same as those required for large cell populations. The only difference is the fact that for the growth of a single human cell, over and above the completely basal medium required for the growth of large cell populations, one must add serine.

Over and above these minor metabolic differences between different cell strains, mutability has been observed in cloned cultures with respect to morphology, chromosome counts, drug resistance, virus susceptibility and biochemical activities. Thus, in our laboratory Dr. DeMars has found a subclone of the HeLa S3 clone which is unable to convert glutamic acid to glutamine even at high levels of glutamic acid. In this specific instance, the block consists in the inability of the cells to adapt to glutamic acid Similarly, none of the cell lines we have examined can hydroxylate phenylalanine to tyrosine. However, an altered strain of HeLa which had become fibroblastic in appearance was now able to hydroxylate phenylalanine to tyrosine in significant amounts, but not enough for growth; it was still an essential amino acid. Six months later, although the cell was still fibroblastic, and still not a typical HeLa cell, it was no longer able to hydroxylate phenylalanine

A third aspect of these cultures which is relevant to the problem under discussion is the disturbing fact that most of these cell cultures do not carry out the specialized functions which are characteristic of the organ from which the cell was derived. In general, fibroblasts do not make collagen, liver cells do not hydroxylate phenylalanine to tyrosine, and do not store large amounts of glycogen. This has obvious relevance to the problem of using cell cultures for the study of biochemical disease or differentiation.

The fourth point is the fact that although cells from established cultures will clone with 80–100 per cent efficiency in essentially the same minimal medium which will permit the apparently indefinite propagation of large cell populations, the cloning efficiency directly from tissue is generally extremely low. It follows that it is impossible to identify with certainty the cells which grow out of culture. Thus, there are available a number of cell lines which are called liver cells. This means only that these cell lines derive from liver, but says nothing as to the identification of the cell type.

Ceppellini: Is it possible to transfer from the tissue culture to the *in vivo* organ?

Eagle: This is perhaps not directly relevant to the topic under

discussion, but a highly significant observation was made by Foley and Handler about two years ago, which has not received the recognition which I think it deserves. There have been a number of reports that cultured mammalian cells will form a tumour on injection into an irradiated or cortisone-treated rat. This is perhaps not surprising. The cortisone-treated or irradiated rat is essentially a culture medium, in which the normal host defences which result in the death of cells have been largely suppressed Foley and Handler found that if cells were injected into the cheek pouch of the normal unconditioned hamster they would grow to form tumours. Even after years of cultivation cells deriving from normal and malignant tissues differed in this respect. In order to produce a tumour it was necessary to inject 100,000 or a million normal cells, while 10 or less malignant cells sufficed. Over and above this extraordinary quantitative difference, there was also a qualitative difference, in that the tumour produced by the normal cells would retrogress spontaneously and was not invasive; while the tumour produced by the malignant cells did not retrogress, ulcerated and was invasive. Although cells do change profoundly in culture, it seems clear that these changes do not necessarily involve a change to a "malignant" state.

To return to the question of why we have this surprising metabolic uniformity in cell cultures and why most cells lose their specialized functions, there are at least three possible explanations. One is that when we grow a cell culture from, e.g. a bit of liver, we begin with an obviously heterogenous population including hepatic cells, bile duct cells, vascular epithelium, Kupfer cells, and there may be differences in the tubular epithelium itself. Only a fraction of the cells may be able to grow: and that selective process may involve the loss of the cells with specific functions. It is, however, possible that the culture is no less heterogeneous than the parent tissue, and that the functions are not evidenced, because we do not provide in the oversimplified media which we necessarily use, precursors or cofactors which are essential for the specific function characteristic of the cell we had hoped to cultivate.

Finally, it is possible that in culture the metabolic machinery of the cell is devoted to growth rather than to function, i.e. the enzymes necessary for function are being formed less rapidly than the enzymes and cell components necessary for growth.

To come back to the major problem of this symposium, even in the present state of the art these cultures lend themselves to the study of many of the problems in human genetics outlined by Prof. Pontecorvo and Prof. Cavalli-Sforza. I do not, however, think that as of now they can be profitably used for studies relating to heritable biochemical disease, or differentiation.

Kalckar: I wonder whether that tumour which was able to hydroxylate phenylalanine may have stemmed from a carcinoid. These carcinoid tumours are very rich in enzymes catalysing the hydroxylation of phenylalanine. Concerning Dr. Eagle's temporary pessimism in regard to the study of inborn errors of metabolism in tissue culture, I suppose it is meant for the next ten years rather than the next 200 years.

Eagle: Your guess there is just as good as mine. There are three major hurdles. We must learn how to clone from tissues with a high degree of efficiency, so as to be able to identify the cell types; we must learn to grow cells from specialized organs which continue to carry out the biochemical reactions peculiar to that organ, and finally we must stabilize the cell with respect to both karyotype and function.

Kalckar: As regards the inborn errors, I was most interested to hear your report about the hydroxylation which shows us how involved the situation can become.

Favism still remains a big question mark. Concerning galactose, I was interested to hear that galactose can replace glucose, as a fuel for cell cultures. Drs. Bentley Glass and Masuo Kodani and I are trying to obtain leucocyte cultures from galactosaemic subjects. We hope that, starting from a galactose-positive population, we may be able to determine between 1,000 and 10,000 cells, using tracer techniques. The idea is first to try our luck on transformation or transduction experiments of galactose negatives by galactose positives. I believe that hereditary galactosaemia at the present time offers a special opportunity.

Pontecorvo: Is this a programme, or do you already know that cultures from galactose positives and galactose negatives can be differentiated?

Kalckar: This is largely a programme which I venture to bring up, as a note of optimism, in this panel discussion on the importance of cell cultures for human genetics. We have initiated work on human cell cultures, using ^{14}C-labelled galactose.

Luria: The matter of the difficulty of maintaining a specific function of the tissue under conditions of rapid or even not too rapid growth is going to be a big stumbling block. It could be, as Dr. Eagle suggested, that in establishing clones one is selecting against cells with specialized function. This may be due to lack of the stimuli or, more probably, of the proper controlling external elements, hormonal and otherwise, which in turn may affect internal controlling elements. Is anything known from other fields, which might suggest some way to go around this? For example, in the culture of nerve cells cultivation is only possible up to the stage of the

Eagle: Not too accurately—anything between pH 7 and 7·8 gives a reasonably good result. In cloning experiments, pH can be rigorously controlled by placing the open Petri dishes in a CO_2 incubator in which the pH is determined by the bicarbonate concentration of the medium, and the partial pressure of CO_2 in the gas. One should not attempt to do experiments in which pH would be a factor, in stationary and stoppered cultures, because of the wide variation (even within a 24-hour period) of the pH in a closed vessel in which the cells are rapidly elaborating lactic acid.

Harris: Is it conceivable that with really accurate control here you may not get some of these peculiar phenomena?

Eagle: It is conceivable but rather unlikely.

Monroy: In connexion with the possibility of obtaining differentiation in stabilized cultures, it seems pertinent to mention here the experiments of Niu who has been able to induce differentiation of neural and non-neural structures from explants of embryonic ectoderm under the influence of ribonucleoproteins from thymus. Although these experiments have been carried out on primary cultures, it would be worth while checking the possibilities of the method on stabilized cultures.

Ingram: Dr. Eagle, what is known about the survival of the haemoglobin-forming cells in bone marrow cultures?

Eagle: There are a number of so-called bone marrow cultures.

Ingram: Do any of them make haemoglobin?

Eagle: None, as far as I know; and in none of them can the nature of the cell be identified.

Luria: Another aspect of genetical study on cells in tissue culture is one which we may call infected heredity. We have heard earlier of two possible types of changes that may manifest themselves in cultured cell lines: mutational changes, and physiological changes analogous to enzymic adaptation or release of repressions. We must consider next the possibility of transductional changes, i.e. of genetical elements coming from the outside into cells and altering the genetic complement. Unfortunately, for the time being there is very little known about such phenomena in either human or other mammalian cells.

We should consider two types of infective heredity: the transfer of subcellular genetic fragments from cell to cell (which might even occur normally in metazoa), and the transfers mediated by viruses, which we consider as specialized vehicles for the transfer of certain specialized genetic elements. Let us use bacteria as a model. We have in bacteria a number of phenomena of infective heredity. First, the DNA genetical elements, which presumably form the bacterial chromosomes, can be transferred by means of cell extracts in

functional form and can become integrated into recipient cells related to the donor cells. Similar transfers can occur by transduction in which a virus, a bacteriophage, is the carrier of the genetic elements. The possibility of transfer of isolated chromosomal fragments or other genetical elements (if any exist) among mammalian cells is being thought about and probably also explored without, as far as I know, any positive results reported for the time being. As for transfers mediated by viruses, they may be of three kinds: persistent viral infections, in which the virus alters cellular functions directly, as is probably the case in virus-induced tumours; persistent infections, where cells may simply become sources of virus without other changes, and transductional changes, in which the virus acts purely as a carrier of cellular genetical fragments. An important thing to remember is that it may pay to look for such phenomena not only by searching for persistent changes in cells, but also for biochemical changes that may manifest themselves in short periods, for example, between infection of a cell by a virulent virus and its death. In bacteria, we know of some bacteriophages whose genetic material includes the genes controlling the production of certain bacterial surface polysaccharides. The new polysaccharide appears on the cell surface within a couple of minutes after infection. It may be of interest to study mammalian cells recently infected by viruses, not only for the production of virus and viral components, but also for possible immunological changes that may manifest themselves very rapidly.

We really do not know anything about the existence and function of extrachromosomal genetic elements in mammalian cells. It is possible that the study of the replication and the genetic functions of viruses whose genetical material consists of RNA may tell us quite a bit about the existence of properties and function of RNA determinants and about the interplay between nuclear genes and plasma genes or other genetical elements whose specificity may be written in RNA code.

Recent work on bacteria has indicated that there are special mechanisms that prevent the extrachromosomal multiplication of certain genetic elements. For instance, if a bacteriophage which can multiply in a bacterial cell, assumes the chromosomal location that is called prophage, the phage genetic element in the chromosomal location generates a mechanism of specific immunity, and suppresses the multiplication of this genetic element in the cell cytoplasm. This immunity is mediated by the formation of some specific suppressors of the function of certain genes which are required for the multiplication of the virus in the cytoplasm. Phages are not the only examples of such peculiar genetic elements or "episomes", which can either

multiply loosely in the cytoplasm or become associated with the chromosomes.

It seems likely that some such mechanisms are involved in maintaining the genetic balance in animal cells. It would be important to decide whether, for example, RNA viruses may represent bits of unrepressed genetic elements, such as unrepressed microsomes. At least in the case of some of the virus tumours, especially that of Rous sarcoma, it has now become possible to observe *in vitro* transformation from normal chick fibroblasts or other chick cells into cells with the growth characteristic of the sarcoma. Here it seems certain that the transformation of a cell from normal to sarcomatous is not accompanied by primary recognizable chromosomal abnormalities, at least as far as chromosome numbers are concerned. Only when these cells become established lines there appears a variety of chromosomal aberrations. It is possible that either the viral element has functioned from a cytoplasmic, an extrachromosomal location, or that it has altered some chromosomal element by changing its growth-regulating functions without necessarily leading to chromosomal imbalance.

Let us now leave this subject and ask Prof Lederberg to deal with a different topic which has to do with responses of cells to other types of environmental stimuli.

Lederberg: Sir Macfarlane Burnet has provided a genetical speculation and a model of the immune response which may bring immunology into the province of somatic cell genetics (1959, The Selection Theory of Immunity. Cambridge University Press).

Two phenomena must be reconciled and interpreted in any comprehensive theory. One is immunity to foreign substances—the development of specific serum antibodies, and in some cases perhaps also specifically sensitized cells, when any of a variety of "foreign" substances are injected into an animal. The second is specific tolerance, the general inhibition of autoimmunity, to substances already part of, or introduced into the animal at an early stage of its development

One view of the immune response which has been generally adopted is that the antigen specifies the structure of the antibody it evolves. It has been thought either to act directly at the intracellular site of antibody formation, or to generate some copy of its own pattern which then governs this process. A corollary to such *instructive* schemes is that an antibody consists of an otherwise indifferent globulin of common or immaterial amino acid sequence which is then folded in a particular way.

This picture is rather disturbing to a unitarian view of the mechanism of protein synthesis. Much of the discussion at this conference

suppose that each cell included a catalogue of forbidden responses in its antibody-forming mechanisms.*

Ceppellini: But a fraction of juvenile cells is always present?

Lederberg: Yes. Sir Macfarlane has, however, suggested that most, if not all, the diversification takes place in prenatal life. I do not feel this is a necessary condition of the theory, especially as Smith's and Mitchison's work indicates that tolerance can also decay in adult life if an antigen is allowed to dissipate.

Burnet: There is still a rather large difference between what you can do in embryonic life or prenatal life and what is possible in the mature animal, but I would agree with you in general.

Ingram: Perhaps your chemostat might be the circulation of a living animal?

Eagle: Yes, it could be. In general, however, this chemostat principle would have to be adapted to monolayer cultures, since it is more difficult to get cultures started in suspension than in stationary culture. This is best reflected by the fact that the minimum inoculum for a stationary culture is one cell, but the minimum inoculum for a suspension culture, even in the established lines, is of the order of 10,000 cells per ml.

Lederberg: Dr. Eagle, did you mention that you get much slower growth in cultures containing a protein level equivalent to whole serum? This might suggest that the concentration of protein in the normal environment of the cell is one of the factors limiting proliferation and favouring functional differentiation *in vivo*

Eagle: No, if you have a good serum, then a 20 per cent and a 5 per cent serum will be equally effective. In most of our metabolic experiments we have deliberately used dialysed serum in order to get rid of the small-molecular weight components.

Kalckar: Are there unsaturated fatty acids in the plasma?

Eagle. Yes, there are, and we have looked into this, because we had previously found that in the growth of a saprophytic spirochaete, the only function of serum protein was to provide lipid bound to the protein. We did indeed try exhaustively extracted serum, and found no difference. However, this does not exclude the participation of lipid, because no method of extraction we have yet found removes all of the lipid

Kalckar: You cannot replace it by tweens?

Eagle: No, they cannot be substituted by lipids.

Lederberg. What is least certain at present is the number and identity of mutable sites in the stem-line cell. The simplest and most vulnerable hypothesis is of a single "globulin gene" subject to

* Discussion abbreviated The topic has been elaborated in a recent publication: Lederberg, J. (1959). *Science*, 129, 1649.

protein-hapten conjugate. How does your theory of antibody induction account for this difference in response?

Lederberg: This is a problem for any model. There are at least two possibilities: one is that arsanilic acid needs to be complexed to reach its target in the cell, or else to react effectively with that target.

Dr. Grubb has also referred here to a "globulin gene", one which governs the antigenic specificity of globulins. This is perhaps not the same gene hypothesized in my discussion as it should control the common carrier chain, not the stereospecific segment. Porter and others have split rabbit γ-globulins into a homogeneous, crystallizable fraction, and another heterogeneous fraction. The first fraction carries the antigenic specificity and thus may well correspond to Grubb's gene. It may also be the fraction which shows the common N-terminal pentapeptide of rabbit globulins though this is still conjectural. The second fraction carries the specific combining ability of the antibody and should correspond to the "stereospecific segment" of the theory.

Ceppellini: In your experiments the lymph node was stimulated by two antigens, each one carried by different particles. One must take into account the possibility that the antibody-forming cell produces only one kind of antibody because it was stimulated by one particle or molecule. It would be interesting to see what happens when one cell is stimulated by a physical entity carrying more than one antigenic specificity; for instance, one could compare the responses after stimulation with a mixture of A and B salivas or after stimulation with AB saliva (in this case the same molecule carries the A and B groupings).

Lederberg: This particular issue could be discussed at great length. But it is not obvious how it would help us to decide between instructive and elective models. Porter's heterogeneous fraction would be the antigen to use.

Grubb: Is it essential to your hypothesis that the amino acid sequence is different in different antibodies?

Lederberg: It is not really essential to elective theories but it is most consistent with other systems of nucleic control. We could retreat to a genically controlled folding pattern, but why set antibodies apart from other specific proteins unless the chemical evidence insists?

Grubb: Is there not some evidence to the contrary, i.e. that the amino acid sequence is the same?

Lederberg: Yes, there has been for rabbit globulins but this is now quite indecisive in view of Porter's recent results. The "common carrier" may be a single protein in the case of rabbit globulin, a

variety of soluble proteins in human globulin. I am not clear whether globulins from individual genotypes have been studied for their chemical constitution. But this need have nothing to do with the stereospecific segment.

Eagle: Your theory has as an essential precondition that the antigen gets into the cell.

Lederberg: I had not doubted this but the least condition is that the antigen should react with the contained antibody of the cell.

Eagle. Would you consider as a possibility a combination of the theory which you and Sir Macfarlane Burnet propose with the instructive theory in the sense that the specifically orientated clone differs from the other only in its permeability to a given protein?

Lederberg: The main question I have been trying to answer concerns the origins of the information for the variety of proteins that antibody forming cells could make. Your suggestion should be considered as equally applicable to instructive and elective theories.

Kalmus: Have you any estimation of mutation rates?

Lederberg: This would depend on the number of antibodies that have to be accounted for. A mutation rate of one per 100 cell divisions will give some hundred million mutant varieties among the 10 billion immunologically competent cells which have been estimated to occur in the rabbit.

Kalmus: Insects which have very poor immunological responses have to meet similar chemical challenges differently, and they meet them, e.g. by undoubtedly chromosomal mutations against new insecticides in the germ cells. As it is comparatively easy to estimate insect numbers we have some idea about the size of population and mutation rates necessary for a particular resistance to develop. Mostly but not invariably the numbers seem to be fairly large and the mutation rates low.

Lederberg: I am sure that the globulin gene must have a much higher mutation rate than the haemoglobin gene, for example. The mutability of this gene is a necessary element that would have to be evolved for it to function in immunity.

Kalmus. But in fact there is a very high mutation rate?

Lederberg: Yes.

Luria: Clearly, high mutability of such a gene will have an advantage.

Lederberg: Yes.

Ingram: I share your difficulty in believing that a single amino acid sequence can assume a large number of configurations, because you are dealing with rather simple surfaces. There have been reports in the literature that people have taken preparations of gamma globulin

and have apparently transformed them into specific antibodies by heating them with antigen.

Lederberg: Would you like to comment on that, Sir Macfarlane? I wonder if the experiment could be repeated.

Burnet: This was described in some early work by Pauling. At the present time I believe that any serum globulin which is specifically reactive with a definable organic configuration can legitimately be referred to as an antibody. Undoubtedly physical changes might be induced in a globulin by heat or the like which modified its reactivity. But I do not think that is relevant to the problem of antibody production.

THE MECHANISM OF GENE ACTION

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ALL modern theories of gene action begin with the hypothesis, first clearly put forward by Beadle in 1945, that genes control the chemical properties of protein molecules. Popularly known as the "one gene-one enzyme" hypothesis, this theory explained very readily how genes, in determining the structural specificity of enzymes, could exercise control over the metabolic pathways of the cell. Yet even as late as 1954 there were still doubts about the unitary nature of gene function and it was held by many that genes could directly catalyse *biochemical transformations*. Three major discoveries in molecular biology have clarified the situation and have allowed the formulation of a unitary theory of gene action. The first was the demonstration by Sanger that a protein, insulin, had a defined chemical structure as expressed by its amino acid sequence (Ryle *et al.*, 1955). The second was the elucidation in 1953 of the structure of deoxyribonucleic acid by Watson and Crick and the third was the work of Benzer (1955) on the genetical fine structure. Of these, the Watson-Crick structure was, if not the most important, at least the most revolutionary for it provided the basis for coherently explaining gene specificity, gene replication and gene mutation. It is not too much of an exaggeration to say that most work in molecular biology during the past five years has been totally based on the hypotheses put forward by Watson and Crick.

In this paper the molecular theory of gene action will be discussed. Gene replication will not be considered, and it is taken for granted that the evidence for nucleic acids as carriers of genetic information is well known and does not need re-

peating. Instead, the problem of information transfer from genes to proteins will be considered in some detail and it will be shown how the general theory must be elaborated in the light of recent information on gene action.

The molecular basis of gene action

We begin with some remarks on the structure of the genetic material. Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are linear polymers, consisting of nucleotide units joined together by repeating phosphate ester bonds. In general, DNA occurs in the form of a double helix, consisting of two polynucleotide chains, running in opposite directions and helically wound around each other. The chains are held together by hydrogen bonds between paired complementary bases, adenine going with thymine and guanine with cytosine. RNA has so far been found only as a single stranded structure both in cells and in RNA viruses and it appears to have no well defined structure (for recent review see Doty and co-workers, 1959) It is thought that this difference is not relevant to the problem of information transfer, especially in the light of Sinsheimer's recent discovery that a small bacteriophage contains single chain DNA (Sinsheimer, 1959). The essential feature of both nucleic acids is that they are, topologically, one-dimensional arrays of nucleotide bases.

By contrast, protein molecules have a much greater range of structure. Chemical studies have shown that the basic unit is the polypeptide chain. This is topologically unidimensional and consists of amino acids, of which twenty different ones occur, arranged in a definite order and linked together by repeating peptide bonds. Superimposed upon this simple primary structure is a more complex secondary and tertiary structure in which the polypeptide chain is folded and superfolded into a three-dimensional form. This structure cannot be revealed by chemical methods, and can be studied only by X-ray crystallography. Recently, Kendrew and co-workers (1958) accomplished a three-dimensional Fourier synthesis of

myoglobin which reveals the complex, but quite precise, arrangement of the single polypeptide chain. Since myoglobin does not contain cysteine, the structure must be held together by a combination of ionic, van der Waals and hydrogen bonds, but in many other proteins, e.g. ribonuclease, the structure is stabilized by disulphide bonds between cysteine residues in defined positions on the polypeptide chain. Many protein molecules contain more than one kind of polypeptide chain. Insulin has two, linked together by disulphide bonds between cysteine residues, while haemoglobin is even more complex. The molecules contain four subunits arranged in two pairs, each pair consisting of different polypeptide chains. The structure is again held together by weaker forces, there being no disulphide bonds.

The specificity of enzyme molecules depends to a very large extent on this three-dimensional structure. Indeed, it seems clear that in designing the protein molecule, Nature has chosen an instrument of great versatility and flexibility in order to deal with the great range of biochemical transformations catalysed by enzymes. Any theory of gene action must therefore explain how the folding of protein molecules is achieved. This is the core of the problem: how can a one-dimensional array of nucleotide bases control the complex folding and superfolding of the polypeptide chain? It might be argued that the structure which assembles the amino acids is already moulded into the negative of this three-dimensional form. From what we know of the structure of myoglobin this appears topologically difficult if not impossible; in any event, one would still have to explain how the negative was obtained from the linear nucleotide sequence. The only reasonable postulate to adopt is that no further information is required to determine the folding apart from the specification of the amino acid sequence of the polypeptide chain. This idea has been called the Sequence Hypothesis by Crick (1958) and it is the basic idea underlying the molecular theory of gene action. In terms of this postulate, we consider the protein only as a one-dimensional array of amino acids linked in a polypeptide

chain, and it is now easy to see that we could place this array in some congruence with the array of bases in the nucleic acid Thus, the mechanism of gene action is reduced to the following: genetic information is encoded in the nucleotide sequence, a defined length of which corresponds to a polypeptide chain. This sequence specifies the sequence of amino acids which in turn determines how the chain will fold and superfold to give an active protein molecule.

The genetical basis for this theory is provided by the work of Benzer (1957) on the fine structure of the r_{II} gene in bacteriophage T4. In this system, there is no technical limitation to the measurement of recombination frequencies as is the case with many other organisms. It was therefore possible to obtain a highly detailed view of a single gene, and to show that a large number of independently arising mutants were resolvable by recombination and could be arranged in a one-dimensional map. In terms of the classical theory of gene action, in which each gene was at the same time a unit of mutation, recombination and function, each of the mutants would have been thought to have been altered in a different gene. This would have meant that the function of the bacteriophage rendered defective by these mutations would have been a composite of a number of individual enzyme reactions each controlled by a single classical gene. Since the number of different mutations found was of the order of hundreds, this theory was immediately rendered absurd. The results are, of course, readily understood in terms of the molecular theory. Each mutation changes the nucleotide sequence at a defined position on the nucleic acid corresponding to a polypeptide chain, producing corresponding changes in the amino acid sequence and rendering the protein defective.

The nice correlation of the one-dimensionality of the genetic fine structure, the structure of nucleic acid and the structure of the polypeptide chain, is the main basis for the molecular theory of gene action. What other experimental evidence may we hope to obtain to prove it conclusively? In the first place, one of the predictions of the theory has already been

selects one chain: that is, one chain contains sense and the other chain is nonsense everywhere. This cannot be done with comma-less triplets but can be if quadruplets are used (Brenner and Crick, 1958; Golomb *et al.*, 1958). Another way is to postulate that the DNA not only codes for amino acids but also contains nucleotide sequences which are controlling elements, i.e. a special sequence may represent instructions to begin and end translation on a given chain.

This last proposal may not be trivial in view of some recent findings on bacterial DNA. It has been known for some time that the guanine to adenine ratio in DNA isolated from different bacteria varies over a wide range from 0·5 to 2·5 (Ki Yong Lee *et al.*, 1956; Belozersky and Spirin, 1958). All of these nucleic acids have adenine to thymine and guanine to cytosine ratios of unity and thus obey the Watson-Crick complementarity rule. In the same bacteria the composition of RNA is almost constant. These are very disturbing facts. One might have expected that if the DNA was a coded system of bases its nucleotide composition would be relatively constant. There are three possible explanations for these facts.

(1) There is no uniform DNA code throughout Nature. The RNA code for amino acids is constant but each bacterium has its own special DNA code. This would mean that each bacterium has its own special translation mechanism, and this does not seem very likely.

(2) There is a uniform DNA code but it is binary, i.e. the code is purines and pyrimidines or 6-amino bases and 6-keto bases. This is not likely, since at least five bases would be required to specify each amino acid, and this number becomes eight as a comma-less condition is imposed. It is then difficult to see how such a system can maintain specificity, since the higher the coding ratio, the greater the error rate must become. In any event, a special mechanism is still required to fix the RNA base ratios. One could argue that the RNA is quaternary, with a triplet corresponding to each amino acid, say, and the DNA is binary. This would necessitate two bases of DNA to code for one of RNA. The genetical coding ratio is still

large, but the two-step process would decrease the error rate. This does not seem likely in view of the structural resemblance between DNA and RNA.

(3) The third possibility is that there are special sequences of DNA which do not code for amino acids but are either purely structural components or represent different kinds of instructions. These sequences could be highly variable in base composition while the rest of the code is fixed and constant. There is no special evidence for or against this hypothesis.

All of this serves to show that the coding problem is not susceptible to an easy solution. Moreover, we may expect that many of our current ideas are probably oversimplified. The experimental attack on the nature of the code is one of the most challenging for current problems in biology.

Control of the rate of protein synthesis

The molecular theory of gene action provides a coherent explanation of the specification of the structure of protein molecules. We may now ask whether the rate of synthesis of a protein can be directly controlled in the same way. Recent work on enzyme biosynthesis in micro-organisms has shown how the molecular theory can be elaborated to include this control. The experimental facts may first be briefly recounted. It is a well known phenomenon that the biosynthesis of many different enzymes in micro-organisms can be induced by small molecules which act specifically. Recently the related phenomenon of enzyme repression has been well studied. This is the counterpart of induction, by which small molecules can repress the synthesis of particular enzymes. Again, repression can be very specific, and not merely due to an overall inhibition of protein synthesis (Vogel, 1957*a*; Gorini and Maas, 1957). A recent ingenious experiment carried out by Pardee, Jacob and Monod (1958) suggests very strongly that the inducer acts by relieving repression of enzyme biosynthesis and that the active process is repression and not induction. The cardinal feature to note is that the rate of synthesis of a

specific protein can be controlled by a small molecule. Where does it act? *A priori*, there are three possible levels where control might be exerted. The first is at the level of the enzyme itself, i.e. an inactive precursor protein is formed which is converted to an active state by a small molecule. This is a trivial case and, in any event, has been excluded in all well studied cases of induced enzyme biosynthesis. The second possibility is that repressors act at the level of synthesis of the RNA templates. This has been neither proved nor disproved, but if such control is exerted it is unlikely to be directly on the RNA itself but probably through the mediation of a protein molecule. The third and most likely level is that of the amino acid assembly into a polypeptide chain. Vogel (1957*b*) has proposed a theory in which the repressor acts by controlling the detachment of the protein from the template. The theory to be discussed below differs in some respects from Vogel's theory, mainly in its greater generality.

It is assumed that the polypeptide chain folds up as it is synthesized, so that as each new amino acid is added, the chain passes progressively through a range of different three-dimensional structures. This postulate helps to explain how the folding is achieved in a spontaneous manner, since, at each stage, the degrees of freedom of the structure are limited. This, of course, would not be the case if the entire chain were *first made and then folded* up. We now assume that at any one stage of synthesis, the folded molecule assumes a particular configuration which blocks the addition of the next amino acid. Clearly then, the rate of synthesis of the complete protein will depend on the probability with which the system can escape from this configuration. A repressor molecule, by combining with a partially synthesized chain, may force it into such a configuration; an inducer molecule may either competitively replace the repressor or act directly on the protein. The important point to note is that the rate of synthesis need not necessarily be controlled by inducers and repressors which are special, sophisticated instances. The theory can explain how many proteins which are not suscept-

ible to induction or repression can be produced at a characteristic rate. All that is required is that a configuration is attained which determines the rate of addition of an amino acid at the growing point. We can therefore make the prediction that the rate of synthesis of a protein molecule is a function of its amino acid sequence and is therefore under mutational control. For repressed enzymes, a change in the rate of steady state synthesis of the enzyme could be produced either by a change in the amount of repressor produced, or by a modification of the protein which would render it more or less susceptible to repression. Furthermore, if it is assumed that economy in protein synthesis is advantageous to the cell, then there will be selection for those amino acid sequences which allow an optimal rate of synthesis but are still consistent with the functional integrity of the completed enzyme.

Cistrons and complementation maps

By the elegant application of the Lewis *cis-trans* test, Benzer (1957) was able to give an operational definition of the genetical unit of function, which he termed cistron. Two r_{II} mutants were placed in the same cell; when the wild type phenotype was observed, the two mutants could be assigned to different cistrons. In this way, a large number of r_{II} mutants were divided into two classes, such that any member of one class could complement any member of the other. The two r_{II} cistrons were contiguous on the genetical map and only the deletion mutants overlapped from one cistron into the other.

There is a simple interpretation of these results. Each r_{II} cistron controls the synthesis of a single polypeptide chain. Each of these polypeptide chains may then form an individual enzyme molecule so that the intact function of the whole region would depend on the successful completion of two separate biochemical steps. Alternatively, the two chains could combine to produce a single protein molecule which performs one biochemical step. The latter is closely parallel

to the findings with haemoglobin discussed by Itano (1959) elsewhere in this volume.

Some recent results on the complementation of mutants in heterokaryons in *Neurospora* and *Aspergillus* show that more complicated situations can exist. An extensive series of experiments reported by Woodward, Partridge and Giles (1958), provides a good example for discussion. These deal with complementation of *ad*-4 mutants in *Neurospora* heterokaryons, the affected enzyme being adenylosuccinase. Of the mutants obtained, over one-half failed to complement with any other mutant. The remainder complemented with at least one other mutant and a large number of different types were found. The relationships of the mutants indicated the existence of seven cistrons which could be arranged in a linear order on the basis of overlapping non-functional regions. Mutants which were functionally defective in more than one cistron always affected contiguous segments. In heterokaryons formed by complementary mutants the amount of enzyme produced ranged from 1 to 25 per cent of that found in the wild type and the latter level was never exceeded.

It is clear that these results cannot be explained on the basis of the model proposed for the r_{II} cistrons. A protein molecule composed of seven different polypeptide chains is not very likely. In any case, it is not easy to explain how mutants which revert spontaneously and are therefore not extensive aberrations can nevertheless have functional effects extending over several cistrons. The suggestion that this type of complementation may represent a physical recombination event at the RNA or protein level is contradicted by the results. If this were so, then the region should approximate to a continuum and not be quantized into a small number of segments. Furthermore, Woodward, Partridge and Giles (1958) have found some combinations to produce a temperature-sensitive enzyme, whereas a recombination process should always give the intact protein structure.

The results may, however, be readily interpreted on the model that the functional enzyme is a polymer, built of

identical subunits arranged in a special way. The present example is best explained by assuming that the enzyme is a tetramer, but the explanation is perfectly general. The assumption that enzymes may function as polymers receives justification in the recent finding of Frieden (1959) that glutamic dehydrogenase is active only in the dimeric state while the individual monomers are inactive. It is also probable that phosphorylase is active as a tetramer (see Korkes, 1956). This allows the existence of a special class of mutants which affect the binding affinity of the monomers, and we may expect cases where two different mutant monomers may have compensatory changes and permit the formation of some functional enzyme.

The adenylsuccinase mutants which are defective in all cistrons could have this property for a variety of reasons. The polypeptide chain may be interrupted and no protein produced; or monomers may be formed which have lost all binding affinity; or the protein may be damaged in some critical part of the active centre. Such mutations could be distributed all over the genetic map. The critical point is whether the linear complementation map corresponds in any way to the linear genetic map. Woodward and co-workers (1958) suggest that this may be the case but the results quoted do not establish it. If there is, in fact, a correspondence, it might reflect the sequential character of the folding process.

The complexity of protein structure makes it unlikely that all complementation phenomena have the same basis. It is worth while pointing out again that in the cases of doubly heterozygous haemoglobin genes, there are no mixed chain dimers formed. This might well have been expected and it suggests that, in this case, the dimers act as units in the construction of the molecule. In a sense, these complications tend to weaken the usefulness of the idea of a cistron when it is defined purely in terms of a complementation experiment. The physical significance of division of a genetic region into cistrons can only be assessed by studying the structure of the corresponding protein molecule.

Conclusions

The fundamental operation in genetic experimentation is the performance of a genetic cross. The existence of the processes of genetical recombination and segregation give us a measure of the genetic material, and allows us to build maps. It may well be asked whether this is in fact absolutely necessary for the study of gene action. It seems clear that, provided mutations are available, considerable information on the molecular basis of gene action can be obtained by the study of the structure of the normal and mutant proteins. The work discussed by Hunt and Ingram (1959) and Itano, Singer and Robinson (1959) has shown what can be achieved in cases where the genetics, by comparison with phage or micro-organisms, is still exceedingly difficult to study. This, of course, is of great importance for the future development of human genetics. There is no reason why the other mutant proteins already known should not be immediately submitted to detailed chemical and physical study. Furthermore, there is no doubt that as screening procedures become more refined more examples are bound to be discovered. Since gene action is undoubtedly the most fundamental of all biological processes no material should be overlooked which can throw light on the problem.

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DISCUSSION

Kalmus: I cannot comment on Dr. Brenner's speculations on protein synthesis because I have no personal experience of the subject; but I should like to discuss some aspects of genetical coding in a way which I have used in a small paper now in press. The analogy between printed and genetical information seems at the moment to fire the imagination of many people and some general considerations seem timely. I should first refer to Dr. Brenner's statement that the frequency of letters in ordinary English texts is very much the same for various authors. This is quite so, but if one compares a higher unit—namely, words—one finds that certain authors use characteristic words much more frequently than others and can be recognized by them. Now let us—very briefly and inadequately—consider how print has evolved historically. It is generally assumed

that natural languages were first spoken before they were written down. How the words, syllables and phonems of any language originated is rather obscure, but chance must have played a considerable part in this coding. Later, at comparable stages in many civilizations objects, actions and situations were drawn for various purposes and from this practice ideographic systems like the hieroglyphs or Chinese symbols derived. These representations are independent of the sound of spoken language, and can thus largely be understood without knowledge of this language. An important and radical departure from the situation was the evolution in one or perhaps two places of a syllabic and later a phonetic transcription. In the resulting systems the majority of symbols do not represent directly the objects which they denote but are transcriptions of the spoken words. The way in which this came about is the central problem of a major branch of linguistics and cannot be discussed here.

The point which I want to make, however, is that it might be profitable to look in the field of genetics for similar hierarchical orders and sequences of codes. It is a distinct possibility that some of the logical difficulties so graphically described by Dr. Brenner may be caused by the fact that direct relationships between two codes have been postulated, when in fact no such relationships exist.

Brenner: This is very difficult; coding is not really like ciphering. We have the problem texts but we do not know what the original language looks like. Suppose one was given a cipher and was not told whether this was captured from the Russian, American, Chinese or Italian Army and asked to decipher it. This would be impossible, if one did not know the original language.

Kalmus: The analogy, of course, has its limitations; but take, for example, the story of the deciphering of the Rosetta stone. The main advance was, I think, made when a special situation was explained, namely the King's name. Further small bits of the code were then broken until the hieroglyphic notation became clear. I think comparable first steps are now being made in the investigations of some of the genetical codes.

Pontecorvo: But two languages were known in the case of the Rosetta stone.

Kalmus: Not entirely; the Greek script was fully understood; the demotic script only imperfectly.

Brenner: On reading the book by Chadwick, "The Decipherment of Linear B," one realizes quite quickly why the nucleic acid protein problem is different. When scholars were presented with *linear B*, they could recognize the semantic context of some of the symbols—

they could recognize horses and women. We are presented with the proteins. Now almost certainly the semantic context of a polypeptide chain of an amino acid sequence has got nothing to do with the original nucleic acid code, but it has to do with the folding of the protein, the structure of the active centres, and so on. In other words, inspection of amino acid sequences can probably tell us nothing about the code, but everything about protein structure. That is what breaking of the protein code would mean in terms of deciphering linear B. We are, however, trying to find the letter congruences between the language of an as yet unknown system, and a system the semantic context of which we do not fully understand.

Lederberg: What do you have against including specific codes for punctuation in the dictionary? Some redundancy in these codes (e g. STOP = ALT = ARRET) might also furnish a place to put the extra guanine you find in some DNA without having to imagine basic changes in the code itself. The common —ACC termination in soluble RNA certainly looks like punctuation.

Brenner: There is no objection to this. One can certainly have codes with a specifically coded comma. For example, consider a binary code in which two of the DNA nucleotides are equivalent so that only two different kinds of letters are used One can show that five letters will be needed to code for twenty amino acids ($2^5 = 32$) and that eight letters will be required if the code, is to be comma-less. We write ABB as a triletter comma, and delete all those five-letter sequences which contain this comma. Of the 32 combinations, 12 are eliminated, leaving exactly twenty! The ABB could correspond to the constant ACC-termination of the soluble RNA, but this would mean that the binary code must be purines or pyrimidines. This code is not to be taken seriously.

Lederberg: If we admit punctuation into the dictionary, could we also use it to convey folding information? For example, the length of the commas and spaces might influence the compactness of amino acid assembly and the degree of freedom for folding angles.

Kalow: I was interested in your ideas on the rate of protein synthesis Does your theory permit the assumption of a variation of protein concentration from person to person, or does it imply that a change of protein structure is necessary in order to produce a change of concentration?

Brenner: No, all the suggestions apply only at the level of the template. Once there is a huge cellular apparatus built on top of this, then there is a further source of individual variation. In the mammalian organism, there are regulatory devices superimposed on the basic system. I do not believe that all these other variations affect the protein structure but, of course, they might.

Itano: The most striking difference between the amino acid analyses of foetal and adult haemoglobins is the presence of isoleucine (about ten residues per molecule) in the former and its complete absence in the latter. Thus, absence of isoleucine could repress foetal haemoglobin synthesis without affecting adult haemoglobin synthesis. On the chance that the presence of a high concentration of isoleucine might induce synthesis of foetal haemoglobin, isoleucine was fed to a patient with sickle-cell anaemia. No increase in proportion of foetal haemoglobin occurred; however, we hope to do further work along these lines with both sickle-cell anaemia and thalassaemia major since in both conditions increased formation of foetal haemoglobin would probably be beneficial to the patient.

Ingram: I do not think that it is very likely to be successful because in the adult cell there is a small amount of other proteins made which do contain isoleucine so that the amino acid is present.

Itano. There may be sufficient isoleucine for some templates but not for others, or the synthesis of isoleucine-containing proteins and adult haemoglobin may not occur at the same time. According to the hypothesis of Dr. Brenner all of the isoleucine positions of each γ chain of foetal haemoglobin would have to be occupied in order that the chain may fold and be released from its template.

Brenner. One could offer a very simple explanation, if one postulated that there is a stage in haemoglobin synthesis when the haems are put onto the molecule before the chain is fully folded. The haem in this case may act as a repressor or inducer. Because the amount of haem or the affinity of the partially folded molecule may depend on the redox potential of the cell one could get an effect of oxygen on the rate of synthesis If the two haemoglobins have different amino acid sequences one could get a differential effect. This is a possibility if both are really in the same cell.

Ceppellini. In regard to the rate of synthesis of haemoglobin Jonxis and Huisman (1958, *Brit. med. J.*, **1**, 1118) have shown that in AB heterozygous sheep, made anaemic through repeated bleedings, the production of Hb B (type I) is favoured at the expense of that of haemoglobin A, i.e. the relative rate of synthesis of the two allelic products is under the control of some factor in the internal environment.

Kalckar: You mentioned the repressor theory. We may have encountered a mutant which although it has a typical inducible β-galactosidase does not contain specific repressors against that enzyme (Kalckar and Kurahashi, unpublished studies).

Burnet: In the normal synthesis of protein, is there any evidence for special gene products which may be found responsible for the secondary folding of polypeptides?

Brenner: The central hypothesis is that all the information to fold the protein is defined by the amino acid sequence of the polypeptide chain. This allows a simple congruence between the sequence of nucleotide bases and that of amino acids. The finished protein molecule has a far more complex structure. The specificity of repression implies immediately that the site of action of the repressor molecule must be at least as elaborate as that for a specific substrate. Such a site could not be designed with a nucleic acid chain nor with a simple polypeptide chain. It must be three-dimensionally patterned and hence *it is perfectly natural to postulate that it is in fact the* folded polypeptide that interacts with the repressor. Repressors and inducers are similar to substrates and products of the enzyme but exceptions can always be found, therefore it cannot be the complete enzyme that reacts and this leads us to suggest that it is an incomplete one that contains the active site for repression or induction I believe that cases may be found of purely inducible enzymes which are insensitive to repression; but so far all cases studied are repressible systems in which the inducer probably acts by competing with a repressor.

Sir Macfarlane Burnet's question can be answered positively. In the cases of tryptophan and arginine biosynthesis the repressors are some derivatives of the amino acids, they act, however, at earlier stages in biosynthesis. Hence in this case a product of one gene can affect the folding of a polypeptide chain produced by another. This case is probably trivial since we are dealing with small molecules. It would be interesting to ask whether large molecules can alter the folding patterns of other molecules. Again, this probably occurs when two proteins pack together to make a more complex structure, but again this is a trivial case. There is no evidence that a polypeptide chain, the amino acid sequence of which is determined by one gene, has its folding directly controlled by another gene, without the intervention of small molecules.

The essence of the argument here is that it is sometimes advantageous *to have amino acid sequences which give rise to ambiguities* in the three-dimensional structure of the protein It is these ambiguities which allow the possibility of control, permitting small molecules to switch one structure into another. Since this property is basically one of the amino acid sequence, it can be changed by mutation, and this type of variability in the protein molecule can be selected for if it is advantageous. Bacteria seem to have specialized in selecting for repressible enzyme systems; we do not, however, know how far this extends to higher organisms.

GENERAL DISCUSSION

Neel. When Prof. Montalenti did me the honour of requesting me to open this summary discussion of the symposium, I was very grateful that the request had not come earlier. Had it come at the outset, I would have felt obliged to take detailed notes and produce something reasonably profound. As it is, the timing of the request gives me some licence to make my remarks both brief and general.

This has been an exciting meeting. I cannot recall attending any other meeting in recent years with such a high content of new material. Couple with that the warm hospitality of our hosts, and we have a remarkable occasion I am enjoined to look a bit into the future. At the moment, one of the very shining possibilities is that some day we may again return to Naples for a similar profitable time

Perhaps the outstanding characteristic of this symposium on human biochemical genetics is the extent to which the basic contributions under discussion have been made by non-geneticists. I am sure that Drs. Kalow, Kalckar, Itano, Ingram, Smithies, Morgan and Watkins will take no offence at being so designated. It is plain that this will continue to be the case for some years to come. There is obviously a fantastic amount of work yet to be done on the haptoglobins and the haemoglobins and the blood group substances, to mention only three of the genetic systems that have come under discussion. The tempo of discovery is truly amazing. In 1947, in a review paper on the detection of the genetic carriers of inherited disease, I could find only three or four reasonably good examples of biochemical carrier states (Neel, J V. (1947). *Medicine*, 26, 115).

This year Hsia has published a book devoted to the inborn errors of metabolism in which he must list at least 20 such states, the exact number depending on how one defines a carrier state (Hsia, D. Y. (1959) Inborn Errors of Metabolism Chicago: Yearbook Publishers). It requires no clairvoyance to see that we have yet to reach the crest of the wave. With the current development of microtechniques, it is quite possible that we shall soon learn the precise basis for the enzymic defect in galactosaemia, deficient cholinesterase, and primaquine sensitivity. Certainly in the abnormal haemoglobins the way is clear not only for the precise identification of genetic changes in the haemoglobin molecule, but to tackle the equally basic question of how many loci contribute to the formation of a single protein, and conversely, in the elaboration of how many

proteins may a single locus participate. The same remarks apply of course to the transferrins of plasma protein.

The realization that now we can test in Man theories of the mechanism of gene action such as Dr. Brenner discussed is every bit as heady as the wonderful nectar we were drinking last evening. These developments pose some very real problems for those of us engaged in the training of geneticists. Would our students be better off if, after an introductory course in genetics, we turned them over to the biochemist, the pharmacologist and the physiologist? Certainly, this could be to their immediate gain; I am not sure that it would be to their long-term advantage. The reasons for this belief are apparent when we take a look at the content of this symposium. Although a number of the genetic systems we have been considering grew out of patient-oriented studies, consider such systems as the transferrins and the haptoglobins, where we really have not more than faint clues to their biological function. Like the well known orphan viruses looking for disease, so we now have a growing number of genetic systems looking for functions. The gene frequencies in these systems are such that something more than simple mutation pressure must be invoked Looking ahead, then, we may expect that as the significance of our ignorance of the function of these newly discovered systems sinks in, we will be driven to both large-scale and very detailed pedigree and population analysis, in our efforts to define the functions of these systems The work on the Lewis-secretor-ABO systems illustrates very well the possibilities for testing in Man, by extensive pedigree studies, postulates concerning a complex genetical and physiological system. A few years ago it was impressive to consider that no two of us were alike serologically. To me it is now equally impressive that any two persons in this room probably differ in half a dozen biochemically definable ways, the biological significance of which in terms of those basic genetic parameters of birth and death we understand not at all. The biochemist will continue his discoveries despite anything we geneticists do—while some of us will find our greatest excitement working at his (the biochemist's) level, the future will also see great activity on a clinicogenetic level as the gap between the test tube and the intact organism is bridged

I hope these remarks are not construed to indicate that I see the day coming when such is the specialization in genetics that one must choose which of numerous compartments will be his scientific abode. Actually, the interrelations of our expanding knowledge are such that we should avoid this, as far as possible, at all costs. A single example of how our genetic knowledge ties together will suffice. For me, it has been a pleasure to attend a meeting where not once has there been mention of the genetic effects of radiation on human

populations And yet, much that we have discussed here has profound implications for this problem. As you know, there is a great debate raging concerning the extent to which human populations are "buffered" *against* the genetic effects of radiation by genetic homeostatic systems. One way of assessing the importance of these systems in Man is through the study of consanguineous marriages. However, the final interpretation of the significance of the results of consanguineous marriages is tempered to a very large extent by knowledge of the allelic structure of human genetic loci. It makes a tremendous difference in the interpretation whether one postulates an average of 3 or 23 alleles for each genetic locus. In 1957 I pointed out the extent to which current biochemical techniques, culminating in Dr. Ingram's precise methods, permitted us to detect previously "invisible mutations", mutations invisible to our dim medical vision but perhaps not so invisible to Mother Nature (Neel, J. V. (1958). Nat. Acad. Sci- Nat Res. Counc., No. 557, p. 253). Here, as well as in some of the serological systems, are outstanding opportunities to contribute data of wide applicability—assuming that as a result of the proper, old-fashioned pedigree studies—supplemented but not replaced by appropriate biochemical studies—we can decide how many loci are involved in haemoglobin production. In this connexion, there seems to be a need for defining a new critical ratio: for each genetic theory there should be at least *one family study* capable of shedding critical light on the theory.

So far no mention of tissue culture. It is obvious that the *potential* contributions of this technique to human genetics could usher in a new era. After the discussion of this morning, we will agree that the millennium is not quite at hand. However, formidable though the problems may be of increasing the efficiency of clonization techniques on fresh tissue to the point where cell selection is not a prohibitive factor in generalizations concerning cell function, of maintaining cell function in culture, and of stabilizing cultures, these problems will undoubtedly be met with time. Who can say what limits there will be to the offspring of the union between tissue culture and biochemistry, or tissue culture and serology? To mention only one of the many basic issues rendered approachable, there is the question of the occurrence and importance of somatic crossing-over and segregation in Man mentioned by Prof. Pontecorvo. I well remember how, as a very new graduate student, I was asked by Dr. Stern to prepare a summary for *Biological Abstracts* of his very classic and equally long paper on somatic crossing-over and segregation in Drosophila. Now, a little over 20 years later, there is the possibility of approaching this same problem in Man. Again, we should recognize into what specialized by-ways these investigations will lead.

But also again, the compartmentalization which I mentioned earlier and which can also be seen here can only be fleeting, since beyond doubt the discoveries to be made on the roller tubes or culture plates, or by whatever techniques are in vogue at the time, will drive us straight back to the original source who, let us as good geneticists hope, has been biopsied rather than autopsied.

Brenner: People who work with haptoglobins should think very seriously of converting directly to finger-printing.

Smithies: We have been contemplating doing this

Brenner: One should not be deterred by technical difficulties. We can expect tremendous advances in techniques. Since the discovery of the genetical code is the most challenging problem of biology, nothing that can offer any information should be overlooked. Man is a large mammal with quite a lot of protein. With the more or less efficient and widely dispersed screening mechanism of clinical investigation, mutant proteins may be found quite readily. Information on the chemical structure of such proteins will be very valuable.

Smithies: One of the most exciting aspects of our work with the serum proteins is that we have not been able to see any abnormalities associated with the various genetical forms of the haptoglobins and transferrins. So if one uses abnormalities to search for differences many variations which could be of wide biological significance might be missed The genetical forms of haemoglobin were originally found as a result of an abnormality, but the red cell blood groups, for example, do not appear to be associated with any abnormality.

Ingram: This will be covered by an extension of our examination of the abnormal haemoglobins to include a survey of the normal ones.

Pontecorvo: How laborious is the detection of differences between haemoglobins which do not determine a pathological syndrome? If you were to collect haemoglobin from 100 individuals, would it be a major operation to identify a few differences and then examine in detail the few unusual haemoglobins?

Ingram: It is not a major operation if the defect falls into the 40 per cent of the haemoglobin molecule which we plan to examine, or if it is an electrophoretic one. It is a major operation if one has to examine the whole of the molecule for each specimen.

Itano: Sanger was able to obtain a unique amino acid sequence of the insulin molecule of any particular species of animal using samples which were pools obtained from many animals. His results suggest that the amount of variation within a species is very small.

Brenner: This raises the possibility of attempting to detect differences in pooled samples. If one knew that in 200 different

haemoglobins pooled together one could, by a very sensitive technique, detect 1 per cent of change, then this would give one courage to go on and look at them individually.

Neel: It is worth emphasizing that no matter which way the analysis of 500 unselected haemoglobin specimens turns out, i.e. whether you find variability or do not find variability, the results are equally challenging.

Ingram: In this connexion may I ask Dr. Neel or one of the other geneticists whether it is possible to take the data on the rare abnormal haemoglobins that are available, and to estimate the degree of "hidden" variability in haemoglobin which one might expect in a human population?

Neel: My answer is no.

Harris: No, but there is also another strange problem. It has always struck me as very curious that the first two mutants of haemoglobin to be worked out by you, Dr. Ingram, should turn out to be exactly at the same point in the amino acid sequence. Here there is thought to be a locus controlling the formation of a polypeptide chain with a sequence of about 150 amino acids, and the first 2 mutants to be found turn out to involve exactly the same site in this Of the other 5 or 6 which you discussed at this symposium, one has turned out to be very close to this first point and the others also appear to be grouped non-randomly. There must be some good reasons why these points in the amino acid sequences have been picked out Admittedly it might be caused by phenomena at various levels; but one should keep this very much in the forefront of one's mind in further work.

Ingram: The tendency at the moment is to explain this by putting the blame on the natural selection of phenotype.

Harris: Admittedly for S and C there may be something in this, but when you get beyond that to some of the others, selection is largely done blindly by electrophoresis.

Neel: Dr. Crick, in a recent conversation, emphasized the possibility that many of these changes may involve the terminal polypeptide in the molecule, a polypeptide which would not be so essential to the configuration of the protein.

Ingram: This is almost certainly not true for some of them.

Neel: We were talking about the SCG polypeptide.

Lederberg: The term "species specificity" has crept into some of the discussions, particularly of immunogenetical relationships. This may have connoted that different proteins from the same organism or species are likely to have a common mark which distinguishes them from the set of proteins of another species. As far as I know this is not so, but I would ask for contradiction. On the contrary,

one would suspect that homologous proteins will show tangible resemblances regardless of species, for example the related amino acid sequences of various insulins.

Ingram: The picture is somewhat complex, but you have both similarities and differences between the haemoglobins of various species at the finger-print level in terms of peptide sequence.

Lederberg: This clearly is a locus specificity. Has the sequence of, say, haemoglobin anything in common with the sequence of any other human protein, such that one would be willing to recognize the set of proteins as having come from the same species? This is what the serologist used to mean when he talked about species specificity. Is it a real concept?

Brenner: I don't think so, because if you take the known sequences of different proteins from the same organism and look at the distribution of dipeptides, these look rather random. I think that *a priori* it is very unlikely.

Itano: The similarities in properties and function of haemoglobin and myoglobin are such as to suggest that the genes controlling the synthesis of myoglobin and the chains of haemoglobin have a common origin. It would be of interest to compare these proteins chemically for possible similarities in positions or sequences of amino acids. Particular attention could be paid to proline because of its unique rôle in peptide bond structure.

Brenner: Whale myoglobin does not look like either horse haemoglobin or human haemoglobin

Ingram: But you can also say that horse myoglobin and horse haemoglobin show distinct differences, both by finger-printing and, for example, in cysteine content. There may be similarities elsewhere.

Kalckar: Since we are discussing the species question, it may be relevant to refer to Tuppy's investigations on cytochrome *c* [Tuppy, H. (1957) *Z. Naturf.*, 12*b*, 184] He has identified the amino acid composition of the haemopeptide of cytochrome *c* from various species.

Smithies: Is it not the generally held view that many groups of proteins with a similar function (e g. haemoglobins) probably arose by evolution from a single "ancestral" protein, so that one would expect to find close similarities in the structure of a particular type of protein even in different species? Evolutionary changes occurring after species differentiation would be expected to produce more or less limited interspecies differences depending on the evolutionary time at which the division into species occurred.

Montalenti: We have come to the end of this symposium. We have all enjoyed the papers and discussions, and each one of us has

learned many things. In a way we may say that the recognition of what we still do not know in this field is more important than the survey of what we know today. It is important and urgent that the work in the field of Human Biochemical Genetics be fostered in each country, by training research workers and giving them the opportunity for investigation in this field, which is so important both from the theoretical and from the practical points of view. I hope that everybody here is going back home with such a programme in mind.

I should like finally to express my warmest thanks to all who have contributed to the success of this meeting by their papers and in discussion.

INDEX OF AUTHORS

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